HISTOPATHOLOGICAL STUDIES OF *BACILLUS THURINGIENSIS* VAR. *KURSTAKI* ON THE LARVAL MIDGUT EPITHELIUM OF *PIERIS CANIDIA* L. AND *PARNARA GUTTATA* BREM. (LEPIDOPTERA)

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LAM Yui-fong, Edmond
Department of Biology

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ABSTRACT

Spore-crystal mixture (2.5%, w/v) of B. thuringiensis var. kurstaki (HD-1) (abbreviated B.t.k.) was fed to the larvae of two oriental butterflies, Pieris canidia L., Parnara guttata Brem.. The effects on larval midgut epithelium were studied at 20 minutes, 40 minutes, 1 hour, 2 hours, 3 hours, 4 hours, and 5 hours after B.t.k. treatment.

Histological studies revealed that, in Pieris larvae, the midgut epithelium was damaged. The columnar cells were readily affected by the B.t.k. treatment. Swelling and regeneration of the columnar cells were observed within the first hour. The newly developed columnar cells were also damaged later. They were swollen and extruded their cytoplasm. Active secreting activities were also observed after B.t.k. treatment. Eventually, most of the columnar cells broke down within four hours. The cellular constituents and some entire nuclei were found in the gut lumen. Goblet cells, on the other hand, were not affected at the the first two hours. The swelling of the goblet cells occurred three hours after B.t.k. treatment. The whole swollen cells then sloughed off from the midgut epithelium.

Histochemical studies revealed that the vesicles from the columnar cells were P.A.S negative, proteinaceous, materials which might be the extruded cytoplasm. These vesicles were released through exocytosis. P.A.S. positive mucous layer was found on the epithelium, which was considered a kind of defense secretion. All the lipid storage in the columnar cells were deprived immediately after B.t.k. treatment. The activities of alkaline phosphatase in the columnar cells dropped in the first hour, increased in the second, and declined again in the fourth hour. The decreased level of enzyme activities might be related to the breakdown of active transport in the original and the newly formed epithelial cells, with eventual disruption of the cells. The ultrastructural changes of the epithelial cells were observed under electron microscope. These results were in concord with the histological changes in the midgut epithelium.

In Parnara guttata, the histological responses of the larval midgut epithelial cells after B.t.k. treatment were different from that of Pieris larvae. The epithelial cells on the Parnara larval midgut were found swollen and increased in vacuolation in the columnar cells. Neither

extrusion of cytoplasm nor regeneration of the epithelial cells were detected in the damaged cells. However, histochemical changes of cellular constituents such as the protein content in the epithelial cells were similar to that of the Pieris larvae after B.t.k. treatment.

In conclusion, the effects of the spore-crystal mixture of B.t.k were primarily on the midgut epithelium of Pieris canidia and Parnara guttata. The differences in histopathological studies of the two larvae may be due to the difference responses in two insects. Moreover, the B.t.k. spore-crystal mixture was found to be highly toxic to both the Pieris and Parnara larvae, in which lysis of damaged cells was observed on or before the fourth hour after B.t.k. treatment. In addition, more than 50% of tested larvae died in the fifth hour.

I. INTRODUCTION

Histopathological studies in the larval midgut epithelium were carried out for the past 30 years in order to find out the mode of action of Bacillus thuringiensis on susceptible insect pests (Angus, 1956; Travers et al, 1976; Endo and Nishiitsutsuji-Uwo, 1980; Oron et al, 1985; Chiang et al, 1986). The silkworm, Bombyx mori (Lepidoptera), an important insect in silk industry, was extensively studied. It was proposed that the oxidative phosphorylation process in the midgut epithelial cells were inhibited by B. thuringiensis delta-endotoxin (Faust et al, 1974; Travers et al, 1976). As a result, the uptake of glucose in the epithelial cells were affected (Fast and Donaghue, 1971) and the mitochondria were found swollen and disintegrated (Ebersold et al, 1977). However, Oron et al (1985) found that the mitochondria in Spodoptera littoralis were not affected by the delta-endotoxin of B. thuringiensis var. entomocidus. They proposed that the primary site of the delta-endotoxin may probably be the membrane of the epithelial cells. Since the insects and the strains of the bacteria used in the two proposals were different, neither of them could exclude the others' hypothesis. In this project, two different species of butterflies, Pieris canidia and Parnara guttata were used to find out the mode of action of B. thuringiensis var. kurstaki on the larval midgut epithelium.

The small cabbage white, Pieris canidia is a common butterfly in Hong Kong and its biology has not yet been extensively studied. It is a close relative to the large white butterfly, Pieris brassica. Pieris canidia is found all year round, especially between March and November (Hill, et al, 1982). Their larvae are serious pests in local vegetables, such as the small cabbage and other cruciferous plants (Hill, et al, 1982). The rice skipper, Parnara guttata, is a common pest on growing rice, it is abundant in Southern China and their larvae cause great loss of rice yield in rainy seasons (Gweneth and Johnston, 1980). The skipper is believed to be the intermediate species between butterflies and moths (Gweneth and Johnston, 1980).

In this project, the histopathological effects of the larvae to B. thuringiensis var. kurstaki were tested at different time intervals in order to find out the sequential responses of the larval midgut epithelial cells after infection. The larval midgut sections of Pieris canidia were studied by light and electron microscopy. However, due to the shortage of material supply, the studies of Parnara guttata were limited to light microscopy. Cellular constituents such as protein, carbohydrates, lipids , and the alkaline phosphatase content were also detected by various histochemical methods after various time treatment intervals.

II. LITERATURE REVIEW OF *BACILLUS THURINGIENSIS*:

2.1) The biology of *Bacillus thuringiensis*

Bacillus thuringiensis is a powerful microbial insecticide in the control of lepidopterous pests. Numerous reports concerning about the toxicity, toxin production, host spectrum, and the mode of action of the *Bacillus* were published. Recently, the genetics of *B. thuringiensis*, the molecular cloning of delta-endotoxin gene and its regulation were intensively studied. (Aronson *et al*, 1986; Whiteley and Schnepf, 1986)

2.1.1.) background

The first report of the bacterial pesticides came from Ishiwata in 1902 (as cited in Aoki and Chegasaki, 1915), who isolated a pathogenic rod form bacteria, which he named *Bacillus sotto*, from diseased silk worm (*Bombyx mori*). Few years later, Berliner, a German scientist, isolated another rod form pathogen from diseased flour moth (*Anagusta knehniella*) (Berliner, 1915). He named it *Bacillus thuringiensis*, which was the first official name of the rod form pathogen. Berliner also reported that the pathogen contained a parasporal inclusion in its sporangium (Berliner, 1915). Unfortunately, he did not pay attention on the parasporal inclusion. Thereafter, a great deal of

investigations were done on the field application of B. thuringiensis. The toxic component remained unknown until the Hannay's rediscovery of the parasporal inclusion in 1953 (Hannay, 1953). The parasporal inclusion was found to be a protein crystal (Hannay and Fitz-James, 1955), which was further identified as the toxic substance for lepidopterous larvae (Angus, 1956). This opened a new page in toxicology research in B. thuringiensis.

2.1.2) the classification and identification

For the growing importance of B. thuringiensis in pest control, many workers were keen on the finding of pathogens for other agricultural pests. As a result, alarge amount of close related pathogens were discovered in the family Bacillaceae. To identify these pathogens, a series of biochemical tests were suggested by Heimpel and Angus (Heimpel and Angus, 1959) to separate different groups of B. thuringiensis and classify them as different varieties of the species. The finding of Ishiwata was then renamed as variety sotto, and the isolate of Berliner was renamed as the variety of thuringiensis. Later, an immuno-assay of the flagella protein of B. thuringiensis were employed to classify the varieties, a series of highly reproducible antigens (H-antigen), was raised to differentiate different serotypes (de Barjac and Bonnefoi, 1973). Until 1981, 19

serotypes were found in B. thuringiensis (Table 1). Apart from the immunoassay, electrophoretic patterns of the esterase produced in the vegetative cells of B. thuringiensis corresponded well with the biochemical and immunoassay methods (Norris, 1964). In addition, the esterases analysis could help subdividing subspecies in the same serotype.

2.1.3) The Physiology of B. thuringiensis

The life history of B. thuringiensis involved three periods: Germination, outgrowing, and sporulation. The nutrient requirement varied in different stages.

a) germination

Most of B. thuringiensis existed in the environment in the form of dormant spore. Under normal condition, the spores were undergoing aging, which involved physiological maturation of the spores. The aging process could be accelerated by artificial factors like heat shock, steam treatment, exposure to calcium dipicolinate, to low pH, or to reducing reagents. These treatments broke the disulfide linkage of the spore coat proteins, this altered their three dimensional conformation (Luthy et al., 1982). After aging, the spores were ready for germination. However, the

Table 1 : Subspecies of B. thuringiensis and their serotype (Faust and Bulla, 1982).

H-antigen serotype	variety name
1	thuringiensis
2	finitimus
3a	alesti
3a3b	kurstaki
4a4b	sotto
4a4b	dendrolimus
4a4c	kenyae
5a5b	galleriae
5a5c	canadensis
6	entomocidus
6	subtoxicus
7	aizawai
8	morrisoni
9	tolworthi
10	darmstadiensis
11a11b	toumanoffi
11a11c	kyushuensis
12	thompsoni
13	pakistani
14	israelensis
15	dakota
16	indiana
17	tohokuensis
18	kumamotoensis
19	tochigiensis

initiation of germination needed glucose, alanine and other amino acids as inducers. The initiation process was irreversible, which first involved degradation of cellular consituents (Rogoff and Yousten, 1969). Heat resistance and refractility of the spore were lost, which followed by the release of dipicolinic acids, calcium and spore coat pepitides. Next, synthesis of cellular constituents for vegetative growth took place: first RNA, secondly fatty acids, and then proteins DNA replication started when all the cellular molecules were well prepared (Rogoff and Yousten, 1969). Cell division was the last process and which ended up the germination process (Rogoff and Yousten, 1969; Luthy et al., 1982).

b) vegetative growth

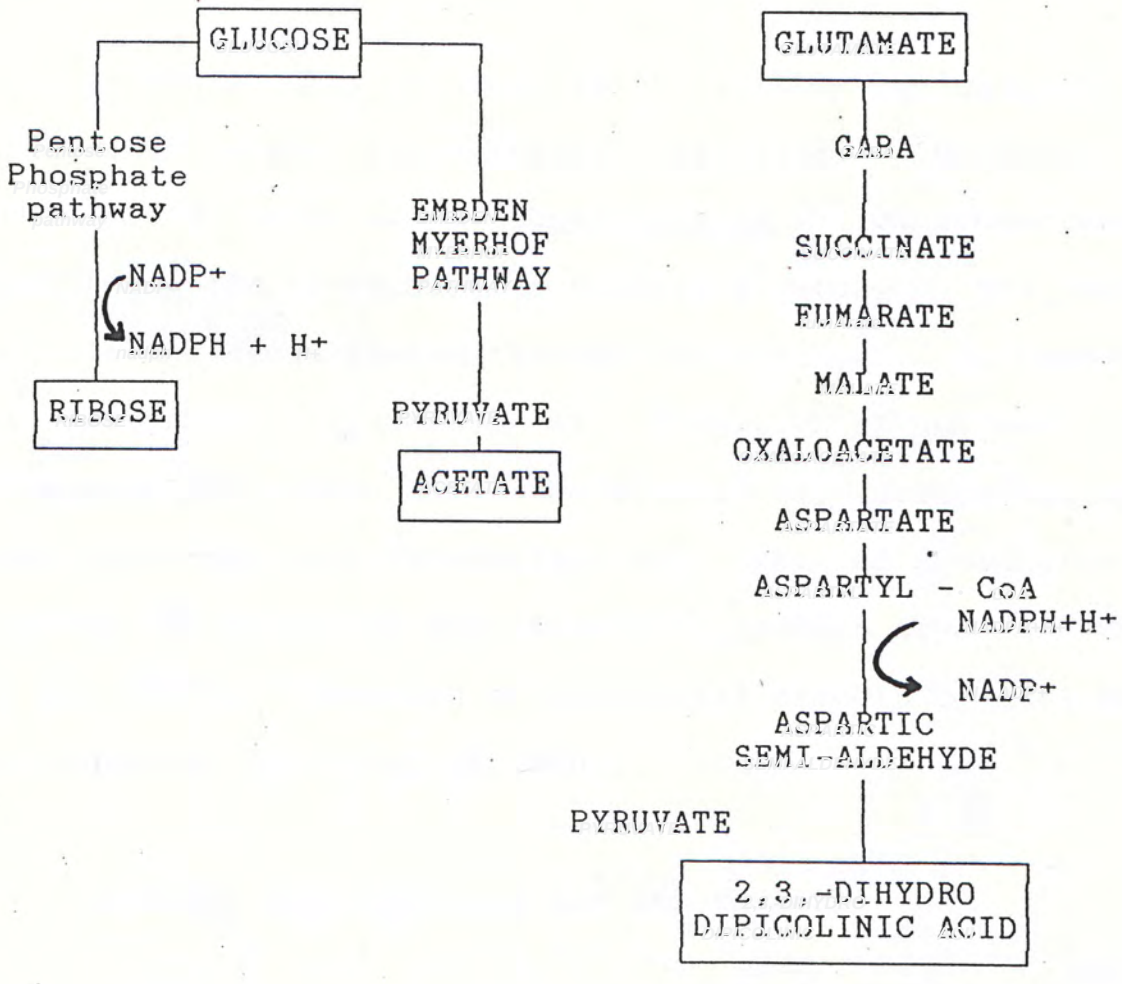
The vegetative cell of B. thuringiensis could not grow in simple, glucose-salt medium. The addition of 2 % glutarate, 2 % aspartate or citrate provided excellent growth for the vegetative cells (Nickerson and Bulla, 1974). Medium supplemented with yeast extract or casein hydrolysate provided excellent growth too, which indicated that, amino acids like glutarate and aspartate were essential for growth. However, unbalance of amino acids in the culture medium led to growth inhibition (Conner and Hansen, 1967; Singer and Rogoff, 1968).

Glucose was the major metabolic precusor for the B. thuringiensis, both the Embden-Meyerhof-Parnas (EMP) pathway (95%) and the pentose phosphate pathway (5%) (Nickerson and Bulla, 1974) took place in the metabolic pathway in the vegetative cell. As a result, pyruvate and acetate accumulated in the culture medium. This decreased the pH of the culture medium, which was unfavourable for the growth of the vegetative cells. Metabolism of glutarate in B. thuringiensis was investigated (Aronson et al., 1975). Because of the absence of α-ketoglutarate dehydrongenase activities in the vegetative cells, and the presence of enzymes in γ-aminobutyric acids (GABA) pathway, It was suggested that glutarate was transformed to succinate via GABA pathway (Aronson et al., 1975). This explained the importance of glutarate in vegetative growth of B. thuringiensis. The metabolic pathway was shown in Figure 1a.

c) sporulation

After glucose had been exhausted from culture medium, the pH decreased as organic acid accumulated. The vegetative cells of B. thuringiensis sporulated. The first metabolic change in sporulation was the utlization of pyruvate and acetate as metabolic fuel. TCA enzymes were found active in the sporulating cells, where activities of isocitrate lyase and malate synthase were found to be

Fig. 1a: The most important metabolic pathway in the vegetative cell of B. thuringiensis



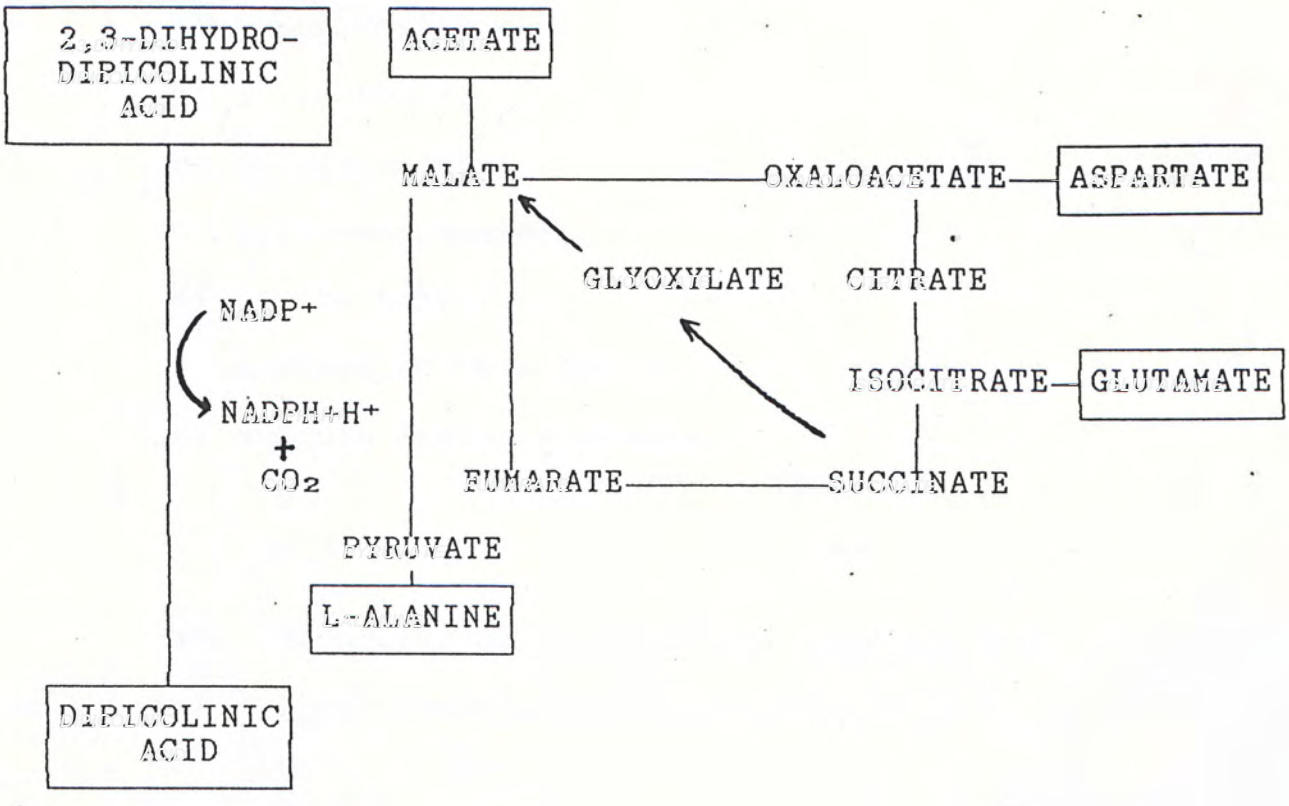
extremely high. However, the lack of α -ketoglutarate dehydrogenase indicated that the TCA cycle was imcomplete (Nickerson and Bulla, 1974; Aronson et al., 1975). It was proposed that glycoxylate by-pass was used to complete the metabolic patway. Indeed, glycoxylate was detected in the sporulating cells. The metabolic pathway was shown in Figure 1b.

The process of sporulation in B. thuringiensis was similar to other spore-forming Bacillus. However, the sporulating cells of B. thuringiensis was characterised by the formation of parasporal protein crystal. The entire sporulating cycle passed through seven stages: I, Formation of the axial filament; II, Formation of septum; III, Forespore formation; IV, Englufment; V, Cortex and spore coat formation; VI, Maturation; VII, Lysis of sporangium and release of endospore and parasporal protein crystal (Bulla et al, 1980). Formation of parasporal protein crystal began at stage III (Bulla et al 1980).

2.2) Bacillus thuringiensis and its toxins

The term toxin referred to 'a poisonous or harmful material obtained from a living organism, or, in bacteriology, a specific class of antigenic poisons of cellular origin' (Faust and Bulla, 1982). Four major criteria were used in the classification of toxins:

Fig. 1b: The most important metabolic pathway in the sporulating cell of B. thuringiensis



1) The anatomical location of the toxin, whether it was secreted outside of the cell (extracellular) or was remained in the cytoplasm (intracellular);
2) Its site or mode of action;
3) Its chemical structure;
4) Symbolic notation.

In B. thuringiensis, seven types of toxins were produced in different strains (Faust and Bulla, 1982). They were:

a) α -exotoxin;
b) β -exotoxin;
c) τ -exotoxin;
d) delta-endotoxin;
e) labile toxin;
f) a water soluble toxin;
g) a mouse factor exotoxin.

The production of insecticidal substances in strains of B. thuringiensis were summerised in Table 2.

Table 2: Production of insecticidal substances by varieties of *B. thuringiensis*. (Faust and Bulla, 1982)

variety name	α-exo-toxin	β-exo-toxin	τ-exo-toxin	δ-endo toxin	other toxins
thuringiensis	+	+		+	Labile exotoxin, Mouse factor exotoxin.
finitimus	+	-		+	
alesti	+	-		+	Water-soluble toxin, Mouse factor exotoxin.
kurstaki	+	-		+	
sotto	+	-		+	
dendrolimus	+	-		+	
kenyae	+	-		+	
galleriae	-	+		+	Mouse factor exotoxin.
canadensis	-	+		+	
entomocidus	-	-		+	
subtoxicus	-	-	+	+	
aizawai	+	+	+	+	
morrisoni	-	+		+	
ostriniae	+	-		+	
tolworthi	+	+		+	
darmstadiensis	-	+		+	
toumanoffi	+	+		+	
kyushuensis	+	-		+	
thompsoni	+	-		+	
pakistani	+	-		+	
israelensis	+	-		+	
dakota	+			+	
indiana	+			+	
tohokuensis	-	-		+	
kumamotoensis	+	±		+	
tochigiensis	+	-		+	

(+, positive; -, negative; blank, not determined.)

a) the α -exotoxin

The α -exotoxin in B. thuringiensis was an exoenzyme, identified as phospholipase C, which was produced by the vegetative cells in the logarithmic growth phase (Toumanoff, 1953). The optimal activities were detected at ten hours after inoculation of the bacteria in the culture medium, with its optimal pH 6-9. The α -exotoxin converted the phosphotidylcholine in cell membranes to diglycerate and phosphocholine. Lysis and necrosis of the target cells resulted.

b) the β -exotoxin

The β -exotoxin was a heat stable toxin, which was secreted outside the cell during the active growth phase. It was a nucleotide-like molecule, water soluble and dialyzable, with the maximum absorbance at 260 nm (McConnell and Richards, 1959). The toxin appeared in a few strains of B. thuringiensis (Table 2). This toxin could effectively prevent pupation in Diptera. The toxin inhibited the RNA synthesis by interferring with the DNA-dependent RNA polymerase. It directly competed with ATP in cellular level and bound to the adenine part of the molecules to the ATP specific site of the DNA complex. Since the binding was

less host specific, some other invertebrates, vertebrates and mammals were also affected by this exotoxin; therefore, it was prohibited in various western countries as U.S.A..

c) the τ -exotoxin

The τ -exotoxin in B. thuringiensis was an unidentifed enzyme, which digested egg yolk agar (Heimpel, 1967). No pathological evidence was obtained from - exotoxin.

d) the delta-endotoxin

The delta-endotoxin was contained in the parasporal protein crystal of B. thuringiensis as an inactive protoxin (Hannay, 1953). After the protein crystal was ingested by the susceptiable insect larvae, Bombyx mori, it was dissolved by the alkaline gut juice. The crystal dissociated and the protoxin was released. The dissolved protoxin was further digested by the protease in the insect midgut to form the active toxin, the delta-endotoxin. The delta-toxin then attacked the epithelial cells in the midgut, swelling and sloughing off of the epithelial cells were then detected (Heimpel and Angus, 1959; Hoopingarner and Materu, 1964). The breakdrown of the midgut epithelium allowed the mixing up of the gut juice with the haemolymph.

As a result, the larvae died of alkalosis (Heimpel and Angus, 1959). Because the delta-endotoxin was highly selective, it was now commonly used as an important insecticide.

e) the labile toxin

The labile toxin was derived from the commercial preparation of B. thuringiensis var. thuringiensis (Smirnoff and Berlinguet, 1966). It was found toxic to the larvae of Tenthredinidae (Hymenoptera). The toxin was very sensitive to air, sunlight, oxygen, and high temperature, therefore, it was called the 'labile toxin'. It was suspected to be an side product of the fermentation process. The mode of action of this toxin had not been determined.

f) the water soluble toxin

The water soluble toxin was isolated from the commercial preparation of B. thuringiensis var. alesti (Fast, 1971). The effects of the water soluble toxin on Bombyx mori larvae was similar to the delta-endotoxin. The molecular weight and the serological evidence showed that the water soluble toxin was not related to the B. thuringiensis delta-endotoxin. Since few investigations were done on these toxin, most of the properties of the water soluble toxin remained unknown.

g) the mouse factor exotoxin

The mouse factor exotoxin was a thermosensitive exotoxin, produced by the active growing cells of B. thuringiensis and B. cereus (Krieg, 1971). It was toxic to mice and several lepidopterans, causing retardation of growth and prolongation of development. The importance of this toxin in pest control was not well established.

2.3) The protein crystal: structure and properties

a) physical structure

The protein crystal was usually bipyramidal or diamond in shape, but it might be cubodial or amorphorus. In bipyramidal crystals, the protein subunits were arranged in stacked sheets which narrowed from the equator towards the poles (Hannay and Fitz-James, 1955; Norris, 1969). The protein subunit was later determined to be the protoxin of delta-endotoxin. Actually each protein subunit were made up of two monomers, with molecular weight 130,000 daltons (Bulla et al., 1977), and of dimension 9.0x9.0x13.5 nm. The two subunits were arranged in a dumb-bell or rod shape (Holmes and Monro, 1965).

b) physical properties

The protein crystal was proteinaeous (Hannay, 1953). To separate the protein crystal from the spores and other residual materials from the culture medium, various methods could be employed, such as gradient centrifugation (Tamir and Gilvarg, 1966; Sharpe et al, 1975), biphasic liquid/liquid system (Goodman et al, 1967), and electrophoresis (Bulla et al, 1979; Bulla et al, 1981). The protein crystal was insoluble in water, acidic and neutral solvent but soluble in high pH, reducing agents and urea. Complete solubilization appeared only in a well formulated solvent mixture of sodium dodecyl sulfate (SDS) plus reducing agent such as β -mercaptoethenol (ME) or dichrothreitol (Bulla et al., 1976). The dissociated protoxin was released in the form of soluble peptide.

c) subunit size

The subunit size of the protein crystal ranged from 1500 daltons to 200,000 daltons (Calabrese and Nickerson, 1978; Bulla et al., 1977). The variation of the molecular weight was probably due to autolysis and protease contamination (Bulla et al., 1977; Chestukina et al., 1978). Two types of proteases, serineprotease and alkaline protease, were separated from the protein crystal

(Chestukina et al., 1978). The subunit sizes of protease-free protein crystal, obtained by NaBr gradient centrifugation, were determined in 16 strains of B. thuringiensis (Calabrese and Nickerson, 1978). They belonged to three categories: Type I, high molecular weight (140 kdal - 160 kdal); Type II, both high and medium molecular weight (60 kdal - 150 kdal); Type III, low molecular weight (60 kdal - 150 kdal). The result of Calabrese and Nickerson was compatible with the other groups (Nickerson, 1980).

d) chemical composition

Generally, the subunit of the protein crystal composed of 95% protein and 5% carbohydrates (Bulla et al., 1977, 1981). Glutamic acid and aspartic acid were the most abundant amino acids in the protein subunit (Bulla et al., 1977), whereas glucose (3.8%) and mannose (1.8%) accounted for the sugars present. Amino sugars, lipids, nucleic acids and sialic acid derivatives were not detected. The protein subunit in B. thuringiensis variety kurstaki, contained 110 amino acids and four sugars (Bulla et al., 1977).

e) insecticidal activities

The protoxin would be digested by the alkaline gut juice and the proteases present (Tojo and Aizawa, 1983). Of all the polypeptides that were released, only the peptides with a definite molecular size was found to have the entomocidal activity (Calabrese and Nickerson, 1978; Bulla et al., 1977). They were called the delta-endotoxin (Heimpel, 1967). However, the entomocidal activity of delta-endotoxin from different B. thuringiensis strains varied, for example, the variety kurstaki contained two types of toxins, the P1 and P2 proteins, of different insecticidal activities (Yamamoto and Iizuka, 1983). The P1 protein was highly toxic to Lepidopterous larvae, the P2 protein was toxic to both Lepidopterous and Dipterous larvae (Yamamoto and McLaughlin, 1981). The variety thuringiensis contained delta-endotoxin, which was toxic to Lepidopterous larvae only. The variety israelensis was found highly toxic against mosquito larvae but non-toxic to the larvae of Lepidoptera (Goldberg and Margalit, 1977). The host specificity depended on the amino acid sguences.

2.4) The delta-endotoxin: its mode of action

a) toxicity

The active component in the protein crystal of B. thuringiensis conferring toxicity was found to be the delta-endotoxin (Heimpel, 1967). After ingestion of the toxin, the columnar cells in the midgut epithelium of silkworm was found swollen, sometimes accompanied by severe vacuolation and extrusion of cytoplasm. The columnar cells then sloughed off from the gut epithelium, as a result, the barrier between gut lumen and the haemoceol collapsed. Gut fluid entered the haemocoel which increased the pH of the haemolymph. The insect was paralysed and died of alkalosis. The actual mechanism of the delta-endotoxin on the midgut epithelial cells was not clear, many of the investigations were done and different hypotheses were being proposed to explain the histological changes in the midgut epithelium.

b) delta-endotoxin as an uncoupler

Glucose uptake increased rapidly in the first minute after the ingestion of protein crystal (kurstaki), by the silkworm (Bombyx mori) larvae. This uptake process peaked at 5 minutes post-ingestion and ceased within ten minutes.

(Fast and Donaghue, 1971; Murphy et al., 1976). The delta-endotoxin was proposed to be an uncoupler of oxidative phosphorylation in the mitochondria (Faust et al., 1974; Travers et al., 1976). The presence of the uncoupler caused the increased of oxygen uptake but inhibited ATP production in the mitochondria, which activated the non-conservative electron transport system. The reducing power decreased as the NADH was consummed. Rate of TCA cycle and Glycolysis was thus speeded up, which yield a high demand of intracellular glucose. The glucose gradient between gut tissue and gut lumen was increased, As a result, a sudden influx of glucose was observed. Intracellular ATP depleted as time goes by. The glycolysis was halted due to the shortage of ATP, which blocked the priming step of glycolysis. Glucose accumulated in the cell and breakdown of glucose gradient resulted. The glucose uptake peaked at 5 minutes and ceased 10 minutes post-intoxication (Travers et al., 1976). The effects of B. thuringiensis delta-endotoxin on the silkworm, Bombyx mori, was compared to other oxidative phosphorylation uncoupler, e.g. valinomycin. The results showed that symptoms in the epithelium were similar after intoxication by both substances. (Faust and Bulla, 1982)

c) as a membrane toxin

Another hypothesis proposed the direct interaction of the B. thuringiensis delta-endotoxin on the cell membrane of the insect midgut epithelium (Fast et al., 1978). They found that the enzyme-activated delta-endotoxin could be covalently bound to the sephadex beams, and that those bindings would probably occurred on the surface of epithelial cells in vivo. However, they lack of conclusive evidence to support this hypothesis. Tests on the relationship between delta-endotoxin and other surface toxins, like cholera toxin, (whose first target was the intestinal membrane) showed that no similarity was found between delta-endotoxin and other surface toxins. Tests were done on the action of delta-endotoxin on sheathed and desheathed cercal nerve and abdominal ganglia 5 and 6 from adult cockroaches (Orthoptera), the membrane conductivity and ion gradient across the axon membrane were not affected (Cooksey et al, 1969). Thus, the delta-endotoxin was not likely to be a surface toxin.

However, the entrance of the B. thuringiensis delta-endotoxin into the insect midgut epithelial cells was not detected, and the initial site of the action was also not determined. There, more works were needed to complete the whole picture of the real mechanism in the action B. thuringiensis delta-endotoxin on insect gut epithelium.

2.5) The histopathological effects of B. thuringiensis on lepidopterous larvae

The histopathological effects of B. thuringiensis on lepidopterous larvae were extensively studied after the reports of Angus and Heimpel in 1959 (Angus and Heimpel, 1959; Heimpel and Angus, 1959). These insects were mainly agricultural pests, such as the wax moth, Galleria mellonella (Hoopingarner and Materu, 1964); larvae of Pieris brassicae (Ebersold et al, 1977; Lecadet and Martouret, 1987); the cotton moths, Spodoptera littoralis, Spodoptera exigua, and Heliothis armigera (Salama et al, 1981; Oron et al, 1985); the tobacco hornworm, Manduca sexta (Delello, 1983); and larvae of the rice moth, Corcyra cephalonica (Chiang et al, 1986). The silkworm, which is a beneficial insect in silk production, is intensively studied as other agricultural pest due to its great importance in sericulture and high infective potency by B. thuringiensis (Fast and Donaghue, 1971; Faust et al, 1974; Nishiitsutsuji-Uwo and Endo, 1980)

The primary site of infection by B. thuringiensis studied so far is confined to the alimentary canal Nishiitsutsuji-Uwo and Endo, 1980). The alimentary canal of all lepidopterous larvae consists of a simple tube leading

from the foregut to the midgut and then the hindgut (Dow, 1986). There are two main cell types present on the midgut epithelium, namely the columnar cells and the goblet cells. The columnar cells are responsible for secretion of digestive enzymes and absorption of nutrients, whereas the goblet cells are for ion excretion, especially the potassium ions (Anderson and Harvey, 1966). The hindgut is the site for water reabsorption and osmoregulation. It is located at the far end of the insect gut.

After infection of B. thuringiensis, the midgut epithelial cells show dramatic changes biochemically and histologically (reviewed by Faust and Bulla, 1982). The administration of delta-endotoxin to the midgut of silkworm (Bombyx mori) caused the cessation of feeding within 15 minutes (Nishiitsutsuji-Uwo and Endo, 1980). The larvae became sluggish and their heart beat decreased to zero in 3 - 4 hours (Nishiitsutsuji-Uwo and Endo, 1980). Ultrastructurally, the columnar cells of the midgut epithelium were damaged within 15 minutes after B. thuringiensis treatment. The basal infoldings of the columnar cells were deformed, and the mitochondria at those regions were in condensed form (Endo and Nishiitsutsuji-Uwo, 1980). The columnar cells then swelled and the vacuole-like structures appeared in the endoplasmic reticulum , the basal infoldings were destroyed within 30 minutes (Endo and

Nishiitsutsuji-Uwo , 1980). The swelling of the columnar cells continued with the microvilli and the basal infolding disappeared and the nuclei swelled (Endo and Nishiitsutsuji-Uwo, 1980). The goblet cavities of the goblet cells were enlarged at 60 - 90 minutes after infection of B. thuringiensis (Endo and Nishiitsutsuji-Uwo, 1980).

The structural changes in other lepidopterous larvae vary according to different authors. In the European corn borer, Ostrinia nubilalis, infection of spore-crystal mixture of B. thuringiensis first affected the goblet cells (Sutter and Raun, 1967). These cells swelled and the microvilli in the goblet cavities disappeared. As a result, vacuolated structures appeared in the midgut epithelium (Sutter and Raun, 1967).

In the larvae of the large white butterfly, Pieris brassicae, the columnar cells and goblet cells were affected by the toxin a few minutes after the administration of purified crystals of B. thuringiensis (Ebersold et al, 1977). Both the microvilli at the apices of the columnar cells and at the cavity of the goblet cells swelled (Ebersold et al, 1977). The mitochondria in the columnar cells inflated and the endoplasmic reticulum disintegrated to form small vacuoles (Ebersold et al, 1977).

However, in the studies of ultrastructural changes in the larval midgut epithelium of the cotton moth Spodoptera littoralis, Oron et al (1985) showed that the microvilli in the columnar cells swelled within 30 minutes after administration of B. thuringiensis spore-crystal mixture, however, the mitochondria were only slightly affected and they remained in a condensed form 40 hours after B. thuringiensis treatment (Oron et al, 1985)

In conclusion, the behavioral responses of the insect larvae to the B. thuringiensis treatment had some similarities. The cessation of feeding and paralysis of the insect larvae were common responses after B. thuringiensis infection (Hoopingarner and Materu, 1964; Ebersold et al, 1977; Nishiitsutsuji-Uwo and Endo, 1980; Salama et al, 1981, Delello, 1983; Chiang et al, 1986). The sloughing off of infected cells from the gut epithelium caused the breakdown of the gut epithelium (Hoopingarner and Materu, 1964; Nishiitsutsuji-Uwo and Endo, 1980) . As a result, gut fluid entered the haemocoel and increased the pH of the haemolymph. The larvae would die of alkalosis (Heimpel, 1967). However, the first target of the B. thuringiensis delta-endotoxin varied in different species. As in Bombyx mori (Nishiitsutsuji-Uwo and Endo, 1980), Pieris brassicae (Ebersold et al, 1977), and Spodoptera littoralis (Oron et

al, 1985), the columnar cells were affected by delta-endotoxin shortly (15-30 minutes) after infection of B. thuringiensis, but in Ostrinia nubilalis, the goblet cells were severely damaged (Sutter and Raun, 1967). The timing of sequential responses were also different in various insect larvae studied. The disruption of microvilli and the other cellular organelles were observed in 15-30 minutes after infection of B. thuringiensis. However, in Pieris brassicae (Ebersold et al, 1977), the swollen of mitochondria were observed a few minutes (5 minutes) after infection. But in Spodoptera littoralis (Oron et al, 1985), the mitochondria remained slightly affected 40 hours after infection. Therefore, the histopathological effected of B. thuringiensis on different insect larvae were quite different, which might vary according to the strain or variety of B. thuringiensis used and the different of insects studied.

2.6) Host specificity, formulation, standardization and safety considerations

a) host specificity

The host spectrum of B. thuringiensis was investigated in various aspects, including vegetable crops, cotton, tobacco, fruit plants, forest, range grasses, ornamental plants, store products, and human diseases vectors (Faust and Bulla, 1982).

The host spectrum of B. thuringiensis was narrow, only insects with alkaline gut juice, pH 8-10, (in which most of the larvae in the order Lepidoptera were) were susceptible to B. thuringiensis delta-endotoxin. Diptera, which composed of most of human diseases vectors, were effectively controlled by B. thuringiensis var. israelensis. It seemed that the host spectrum of the B. thuringiensis in different varieties played an important role in pest management.

b) formulation

The formulation of commercial products of B. thuringiensis was an important criteria in field application. Recently, wettable powders, dusts, granular preparations, and aqueous concentrates were available in the

market. The wettable powder was widely used in controlling foliage-chewing caterpillars whose were easyily contacted by simple spraying of the insecticide. Droplet size, coverage and spraying concentration affected the efficiency of the insecticide. The mortality per unit of application was found inversely related to the spraying rate. The mortality also increased when the droplet size and spray concentration was increased. The use of wettable powder become limited when used in the pest control in corn, whose stem borers entered the inside of the corn stem, this shelthered the insects from direct exposure to insecticides. Granular formulation of B. thuringiensis relieved this conidition, which made the control of European corn borer possible in the first generation. However, the efficacy was reduced in the second generation. (Lynch et al., 1976). Dust preparations provided a more uniform plant coverage, which was found superior to spray formulation in the control of cabbage looper and imported cabbage worm (Pieris rapae) in Southern California, and the control of the semilooper in castor bean. Indeed, the formulation determined the efficiency of B. thuringiensis preparation in the control of agricultural pests.

c) standardization

As the commercial preparation of B. thuringiensis was not a pure stock but was a mixture of spores, protein crystals, inert additives and sometimes the residues of supporting medium, the standardization of the commerical preparation was very important in the reliability of the B. thuringiensis.

The world-wide adopted standardization methods were based on a comparative mortality of the target insects, which represented by International Unit (I.U.) (Dulmage, et al., 1971). Two standards were set for two strains in B. thuringiensis: the variety thuringiensis in Pasteur Institute and variety kurstaki in U.S.A. (Dulmage et al., 1971). The reference standard prepared by the Pasteur Institute contained 1000 IU/mg of protein crystal, which based on the the toxicity of B. thuringiensis variety thuringiensis on Mediterranean flour moth (Anagasta kuehniella). The second standard was set by Dulmage in U.S.A. who used the cabbage looper (Trichoplusia ni) as the target insect. The toxicity was determined by using the strain kurstaki, designed as HD-1-S, having potency of 18,000 IU/mg of the 13000 dal. protein subunit (Dulmage, 1971). The standardization methods mentioned above depended on the result of bioassay. However, the result of

bioassay varied with the physiological condition of the target insects, the heterogenicity of the food. Moreover, the bioassay itself was a time-consumming process; therefore, direct determination of the toxicity in commercial products was being investigated. The immunoelectrophoretic method based on the polarity and immuno-assay of the crystal protein was first found corresponded well with the result of bioassay method (Krywienczyk et al., 1978). The ELISA (enzyme-linked immunosordent assay) method was then applied for the direct determination of the protein crystal content in the commercial products. The result was equivalent to the bioassay (Luthy, et al., 1982). However, the ELISA method in the same B. thuringiensis strain and the result only showed the amount of the crystal protein but did not account for the toxicity of the insecticide. Thus, more investigations were still needed in the direct measurement on the entomocidal activity in various preparation of B. thuringiensis.

d) safety considerations

Safety test was an important criteria for the commercial products of B. thuringiensis, where the inseciticides were sprayed to the open area. Before putting into the market, the effects of these insecticides to the

public and its environment must be tested. Different families of agriculturally beneficial insects, invertebrates, vertebrates, mammals, and human volunteers were being tested (Heimpel, 1971; Faust and Bulla, 1982). The results showed that the B. thuringiensis preparation was non-toxic to the other groups of animals. The possibility of the mutation of B. thuringiensis to a human pathogen was also tested. Tranformation and transduction was unlikely as the DNA donor did not exist in the pure culture of B. thuringiensis. However, the spores will be killed shortly after exposure to sunlight, which prevented any mutations after spraying.

III. MATERIALS AND METHODS:

3.1) Collection and rearing of butterflies

The small cabbage white butterfly, Pieris canidia L., was collected in Lam Chuen and the Chinese University campus in New Territories. Under captivity in a rearing box, measuring 25x25x40 cm, they were supplied with 5% sucrose solution for their daily diet, and a small cabbage (Tsoi Sum) plant, Brassica parachinesis B., for oviposition. It was found that the presence of a suitable host plant was a critical requirement for oviposition. Thereafter, the cabbage with eggs were transferred into an insectarium, in which the temperature was kept at 23 $\pm$ 1°C, and the photoperiod was maintained at 14 hours per day. The larvae hatched from the eggs four to seven days after they were laid. The newly hatched larvae moved to the lower side of the leaf. At this stage, the larvae ate only the plant tissues, leaving the wax layer of the upper leaf surface intact, resulting in small transparent holes on the leaves. Moulting took place within one day and the second instar larvae consumed the whole part of the leaf, so the damages of the plant became severe. If food supply was sufficient, the larval period passed through five successive instars within 13 days . After the fifth moult in larval period, the caterpillars started pupation. Metamorphosis usually completed within one week. After the imagoes had emerged from their chrysalis, they were then collected and put into the rearing box for mating and oviposition.

Adult rice skipper, Parnara guttata Brem., were collected in the field around the University campus during November. They were then put into the rearing box for mating and oviposition. For Parnara spp., oviposition was independent of the host plant but had a strict requirement for temperature above 25 °C. Eggs were collected by putting a sheet of paper or a thin paraffin plate on the bottom of a rearing box. Thereafter, the paper or paraffin plate with eggs were cut into small pieces. They were then placed onto a potted rice plant in the insectarium. The temperature in the insectarium was kept between 25 and 30 °C, and the photoperiod was maintained in 14 hours light period. Four days after oviposition, the larvae hatched. The newly hatched larvae were milky in body colour with a big black head. They bent a rice leaf into a tube with the help of silk. After that, they lived in the tubes and fed on the plant tissue around their 'house'. Moulting took place in the constructed tube, and the newly emerged larvae found new places on the host plant to construct bigger homes. The larval period passed through five successive instars. At the end of the fifth instar, the larvae searched for a safe place for pupation, they hide themselves among the lower plant leaves. Adults emerged from the cocoon within one week. The butterflies were then collected and kept in a rearing box to start the next generation.

3.2) Bacterial strain

Bacillus thuringiensis var. kurstaki (HD-1) was provided by Sandoz company, under the trade name of Thuricide. The bacterial preparation was in the form of spore-crystal mixture, with potency 16,000 Internation Units per gram.

3.3) Insect and inocula

Larvae of Pieris canidia and Parnara guttata in the penultimate instar were starved overnight. They were then immersed into an aqueous suspension of spore-crystal mixture (2.5%, w/v) of B. thuringiensis var. kurstaki, or distilled water for five seconds (Unit of Insect Microbiology, 1977). It is important to ensure the heads of the larvae were completely wetted by the suspension. The treated larvae were then transferred onto a fresh leaf of small cabbage for 20 minutes, 40 minutes, 1 hour, 2 hours, 3 hours, 4 hours, and 5 hours, at which time they were processed by the procedures described below.

3.4) Histology: Light and Electron microscopy

a) Light microscopy

The larvae were dissected in cold 0.2 M phosphate buffer, pH 7.2, with the midgut isolated. The tissues were immediately fixed with Bouin's fluid for at least 24 hours, then they were followed by dehydration through an ascending series of ethanol, cleared in xylene, and finally embedded in 56-58°C paraplast. Serial sections of the entire midgut were cut at 5 um and stained with Hematoxylin and Eosin1. Materials for histological studies were also fixed in glutaraldehyde and post fixed in osmium tetroxide as for electron microscopic studies.

b) Transmission electron microscopy

The removed midgut was fixed with 2.5 % buffered glutaraldehyde for 1 hour at 4 °C (0.2 M phosphate buffer, pH 7.2-7.4). The tissues were then washed with phosphate buffer, then post-fixed with 1% osmium tetroxide for 2 hours at 4 °C. Tissue were dehydrated by ascending series of ethanol in an ice bath, followed by embedding in Spurr's medium. The resin was allowed to polymerise at 70 °C for 12-16 hours. Thick sections of 1 um thickness were cut by a

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1. Please refer to Appendix - 1 for detailed information.

Reichert Om-U2 ultramicrotome and stained with 1 % toluidine blue in 1 % borax for light microscopy [1]. Thin sections were cut and stained with uranyl acetate and lead citrate for observation under Zeiss EM9S-2 electron microscope.

c) Scanning electron microscopy

Pieces of removed midgut were fixed in 2.5 % phosphate buffered glutaraldehyde and postfixed in 1 % osmium tetroxide as in TEM studies. After dehydration in an ascending series of ethanol in an ice bath, the tissues were infiltrated with amyl acetate and dried by CO_2 in a critical point dryer. The tissues were then coated with a thin layer of gold and observed under Joel JSM-35 scanning electron microscope.

3.5) Histochemistry

Histochemical studies were carried out for the following cellular materials:

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1. Please refer to Appendix - 1 for detailed information.

a) Proteins: Insect midgut tissues were fixed by Bouin's fluid for at least 24 hours, dehydrated in an ascending series of ethanol, cleared in xylene and embedded in 56-58°C paraplast. Serial sections of pieces of midgut were cut into 5 um thickness. General proteins were demonstrated by Mercury-Bromophenol Blue method[1] (Troyer, 1980). Basic proteins were demonstrated by Acid Solochrome Cyanine staining technique[1] (Pearse, 1968).

b) Carbohydrates: Serial paraffin sections were obtained as in tissue preparation for protein study. Carbohydrates such as glycogen and other polysaccharides were demonstrated by Periodic Acid-Schiff[1] technique (Culling, 1963). Control was done by incubation of slides in 1% diastase at 37 °C for one hour. Acid mucoproteins were demonstrated by Alcian Blue method[1] (Culling, 1963).

c) Lipids: Insects were dissected in cold phosphate buffer, The entire midgut was removed and fixed in cold formal-calcium for 3 to 4 hours at 0-4°C. Fixed tissues were infiltrated with 30% sucrose solution for 6 hours to overnight, then they were frozen in liquid nitrogen and embedded in O.C.T. medium for cryostat sectioning. Sections of 10 um thick were cut for staining in Sudan Black[1] (Humason, 1972). The distribution of phospholipids were noted.

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1. Please refer to Appendix - 1 for detailed information.

d) Alkaline phosphatase: 10 um thick cryostat sections of insect midgut were cut. The alkaline phosphatase activities were demonstrated by substituted naphthol method (Troyer, 1980). The cryostat sections were incubated in Naphthol AS-TR phosphate in Tris buffer, pH 8.4, for one hour. Before incubation a red azo dye, Fast Red TB salt, was added in the substrate. Thereafter, the tissues were rinsed in distilled water and mounted in 50 % glycerin.

8) Recovery of bacteria from infected larvae

The larvae were immersed into B. thuringiensis spore-crystal suspension (2.5%, w/v) for 5 seconds, they were then transferred to fresh plants for five hours. Thereafter, the infected larvae were dissected and their midguts were isolated and homogenised with 1 ml phosphate buffer (pH 7.2). 0.1 ml of the midgut extract was spreaded on a nutrient agar plate and incubated at 30°C overnight. The colonies on the agar plate were isolated, stained by Gram's method and observed under phase contrast light microscope (Bulla et al., 1969).

IV. RESULTS

4.1) Histopathology of the midgut epithelium of *Pieris canidia*

Throughout the project, adults of *Pieris canidia* (Fig. 1, 2) were caught from the field. They were then allowed to breed and their larvae (Fig. 3) were reared in the laboratory. The fifth instar larvae, shortly after moulting, were used in this project. More than 20 larvae were used for histological and histochemical studies, 10 for transmission electron microscopy, and 6 for scanning electron microscopy at each time interval tested. The results were summarised in the paragraphs below.

4.1.1) The histological changes in the larval midgut epithelium of *Pieris canidia*

A) The normal midgut epithelium in *Pieris canidia*

The alimentary canal (gut) of *Pieris canidia* larvae, like the other phytophagous insects, is a straight tube starting from the head to the last segment. The gut is composed of foregut, midgut and hindgut. The foregut, which

leads from the buccal cavity to the oesophagus and crop, is responsible for the temporary storage of the plant materials ingested. The midgut, where digestion and absorption take place, is the most important organ in the larval gut. It is a straight tube without any superficial differentiation. The hindgut can be divided into three regions, the ileum, the colon and the rectum (Fig. 4).

i) Structure of the midgut epithelium

Under light microscope, the midgut is a thin, mono-layered epithelium which separated the gut lumen and the haemocoel. It is composed of two major cell types:. namely, the columnar cells and the goblet cells. Some undifferentiated cells, named regenerative cells, are found in the epithelium occasionaly. (Fig. 5)

ii) The columnar cell

The columnar cell is a tall cell with a centrally located nucleus. Its apical surface is much folded to form a brush border (Fig. 6). Under electron microscope, it is a layer of closely packed microvilli (Fig. 7). At the basal region, many extracellular spaces are formed by the infolding of the basal plasma membrane. These spaces usually extend 1/3 distance up the columnar cells. Under

electron microscope, mitochondria are found associated with these extracellular spaces, so active transport of glucose, amino acids and ions might take place across the membranes between the cytoplasm and the extracellular space (Anderson and Harvey, 1966). Well-developed endoplasmic reticulum network appear in the cytoplasm with abundant rough endoplasmic reticulum (Fig. 8, 9). Active protein synthesis is speculated to be extensive in the columar cell.

iii) The goblet cell

The goblet cell is another major cell type of the midgut epithelium. It is a specialized cell type with a basally-located cup-shaped nucleus and a large urn-shaped cavity opening at the luminal side (Fig. 6). On the surface of the goblet cavity, numerous microvilli project into the center of the cavity and each of them may contain an elongated mitochondrion (Fig. 10) According to Anderson and Harvey (1966), active transport of potassium ions is speculated to take place in the goblet cavity.

iv) The regenerative cell, the muscle sheath, and the peritrophic membrane

The regenerative cell is a undifferentiated cell on the epithelium (Fig. 6). It is conical in shape and is located near the basement membrane. It has the ability to regenerate into a epithelial cell, such as a columnar cell. The midgut epithelium is enclosed by a muscular sheath, which is composed of circular and longitudinal muscles. The muscle sheath generates force for contraction of the gut. A peritrophic membrane is also detected on the inner surface of the gut wall. On the inner side, a peritrophic membrane covers the apices of the epithelial cells and is found enclosing the food. The peritrophic membrane is secreted from the brush border, and is composed of chitin and lipoprotein materials. It protects the gut wall from mechanical damages caused by the food particles. The surface of midgut epithelium at the luminal side was folded to from crests (Fig. 11).

B) Histological changes in the midgut epithelium of infected larvae

After treatment of B. thuringiensis var. kurstaki spore-crystal mixture, the histopathology of the midgut epithelium in Pieris canidia was studied by light and electron microscopy.

i) One hour post-treatment

One hour after treatment of B. thuringiensis spore-crystal mixture, feeding of the Pieris larvae stopped. The larvae became inert and paralysed. Under light microscope, the columnar cells were seen to be infected with bacterial spores and rods. Swelling of the cells and increase in vacuolation in the cytoplasm were observed. Many secretion bodies were found on the apical region associated with the cytoplasmic projections (Fig. 12). The microvilli were disrupted (Fig. 13). In the cytoplasm, the network of endoplasmic reticulum started to disintegrate, and small vacuoles were observed in the cytoplasm (Fig. 15). The mitochondria were found to be more electron dense (Fig. 16). Extracellular spaces and intercellular spaces were increased in size (Fig. 14). Bacteria were found in the gut lumen, Some were embedded in the peritrophic membrane and some were

found penetrating into the columnar cells. The goblet cells, on the other hand, were less damaged (Fig. 17). Under scanning electron microscope, the surface of the midgut epithelium became rough as rapid secretion and extrusion of cytoplasm took place (Fig. 18, 19).

ii) Two hours post-treatment

Two hours after intoxication, continued secretion was observed in the columnar cells with disruption of microvilli continued. Vacuolation was severe in the cytoplasm of columnar cells. Penetration of bacteria in the columnar cells was observed (Fig. 20, 23). Ultrastructurally, the size of extracellular spaces and intercellular spaces were further increased (Fig. 21), with the infolding of basal plasma membrane extended to 2/3 distance up the columnar cells (Fig. 22). However, the goblet cells were less affected at this stage. The surface of the midgut epithelium was disrupted and many secretory bodies and extruded cytoplasmic vesicles were observed (Fig. 24, 25, 26).

iii) Three hours post-treatment

Continuous damages in the midgut epithelium was observed in the third hour after B. thuringiensis treatment. The columnar cells swelled and the microvilli were completely disrupted. The apical surface of columnar cells was thus smooth. Lysis of columnar cells was observed in some area, where all the cytoplasm was lost and their intact nuclei were left and attached to the basement membrane. Some epithelial cells became independent cells. The goblet cells were slightly swollen (Fig. 27).

iv) Four hours post-treatment

The goblet cells swelled and sloughed off from the epithelium (Fig. 28). Single rod-form bacteria was observed in the gut lumen in the damaged columnar cells and also in the fat body (Fig. 28). The apical surface was further disrupted, numerous cytoplasmic projections and secretory materials were observed on the surface of the midgut epithelium at the luminal side (Fig. 29, 30).

v) Five hours post-treatment

Five hours after treatment, the larvae were completely paralysed and more than 50% of them died. Anatomically, the midgut was highly contracted and brown coloured fluid was observed in the midgut although green plant materials were still found to be stored in the crop. Microscopically, the midgut epithelium was seen to be disrupted and a thin layer of basement membrane was left. The remaining cells were heavily contaminated with bacteria (Fig. 31).

4.1.2) Histochemical studies of the larval midgut epithelium in Pieris canidia

A) Histochemical studies of the normal midgut in Pieris larvae

Four cellular components in the larval midgut epithelium of Pieris canidia were tested by various histochemical methods. They were proteins, carbohydrates, lipids, and alkaline phosphatase contents. The results were reported in the following paragraphs.

i) Proteins: the proteins in the midgut epithelium were determined by Acid Solochrome Cyanine and Mercury Bromophenol Blue methods. Most of the secretory substances of the epithelial cells contained proteins (Fig. 32), which were mainly basic proteins (Fig. 33)

ii) Carbohydrates: The tissues were stained by P.A.S. and Alcian Blue methods. Most of the P.A.S. positive substances were located on the apices of columnar cells (Fig. 34). However, they were not stained by Alcian blue (Fig. 35). The goblet cavities contained secretory bodies which were also P.A.S. positive. These secretory bodies were slightly stained by Alcian Blue.

iii) Lipids: Phospholipids were demonstrated by Sudan Black method. In the normal midgut epithelium, lipid droplets were found to be abundant in the cells. (Fig. 36)

iv) Alkaline phosphatase activity : The activity of alkaline phosphatase was found on the apical surface of the columnar cells. It was believed that the alkaline phosphatases were located on the brush border. (Fig. 37)

B) Histochemical changes in the larval midgut epithelium of Pieris canidia after B. thuringiensis spore-crystal treatment.

The effects of B. thuringiensis spore-crystal mixture on the cellular composition in larval midgut epithelium of Pieris canidia were tested by various histochemical staining methods. The results were listed below:

i) 20 minutes post-treatment

20 minutes after intoxication, rapid extrusion of cytoplasm took place. Large amount of cytoplasm was lost by exocytosis. They were mainly proteins (Fig. 38). However, increase in basic protein content was observed in columnar cells, (Fig. 39). Secretion of P.A.S positive substances were also detected on the apical surface of the columnar cells and within the goblet cavities (Fig. 40). These secretions did not contain acid mucin as they could not be stained by Alcian blue, but being however, it was extensively stained by safranin (Fig. 41). No lipid droplet was detected in the cytoplasm of columnar cells (Fig. 42). The decline of alkaline phosphatase activity was also noted in the columnar cells (Fig. 43).

ii) 40 minutes post-treatment

40 minutes after intoxication, the extrusion of cytoplasm continued (Fig. 44), the basic protein level at the epithelium remained high (Fig. 45). Secretion was found on the epithelial surface, no acid mucin could be detected in the epithelium (Fig. 46,47). Lipid content, however, could not be detected in columnar cells (Fig. 48). The loss of alkaline phosphatase activity was noted in columnar cells in this stage (Fig. 49).

iii) One hour post-treatment

At the end of the first hour, replacement of damaged columnar cells by the regenerative cells were observed. Thus, the size of the cells decreased, but the major chemical contituents of the epithelium were less affected. The P.A.S positive substances remained in a layer covering the epithelial cells (Fig. 52, 53), and the basic protein level in the midgut epithelium was changed (Fig. 50, 51). No lipid droplet could be detected in the columnar cells (Fig. 54). The alkaline phosphatase activity, on the other hand, was observed at the centre of the columnar cells and seemed to be transported to the brush border (Fig. 55).

iv) Two hours post-treatment

The secretory substances were found covering the apices of the epithelium. The columnar cells were swollen, and the pH in the gut lumen remained high (Fig. 56, 57). No lipid droplet could be detected in the columnar cells (Fig. 58, 59, 60). However, The activity of alkaline phosphatase increased and accumulated on the apices of the columnar cells (Fig. 61).

v) Three fours post-treatment

Three hours after intoxication, loss of the secretory products on the epithelium were observed after rapid lysis of the columnar cell (Fig. 62, 64). The goblet cells, in this stage, became swollen, and the uptake of acid solochrome cyanine was observed. The basic protein was abundant in the epithelium (Fig. 63), and the staining of Alcian blue gave negative results (Fig. 65). No lipid droplet was detected in the epithelium (Fig. 66). The alkaline phosphatase was found accumulated in some remained columnar cells (Fig. 67).

vi) Four hours post-treatment

At the fourth hour after intoxication, rapid slough off of the epithelial cells was observed (Fig. 68, 69). Swollen goblet cells were found in the gut lumen heavily stained by P.A.S. reagent (Fig. 70, 71), Acid solochrome cyanine (Fig. 69) and Mercury Bromophenol blue (Fig. 68) Intact nuclei from lysed columnar cells, stained by hematoxylin, were observed in the gut lumen or attached to the remaining substances on the epithelium (Fig. 70, 71). As most of the cell lysed, neither lipid droplet nor alkaline phosphatase activity could be detected (Fig. 72, 73).

vii) Five hours post-treatment

At the fifth hour, most of the cells lysed or detached from the epithelium, only a thin layer of cuboidal cells with less cytoplasm were found remained on the midgut epithelium (Fig. 74, 75, 76, 77).

4.2) Histological and histochemical studies in the midgut epithelium of *Parnara guttata* after treatment of *Bacillus thuringiensis*

A) The normal midgut epithelium

The basic constituents of the larval midgut epithelium of *Parnara guttata* were similar to those of the larvae of *Pieris canidia*. The columnar cell and goblet cell were the major cell types in the midgut epithelium. A striated brush border lined the apical surface of the midgut epithelium (Fig. 78), covered by P.A.S. positive materials (Fig. 79). The basic protein level, however, was found lower in *Parnara guttata* midgut than *Pieris* (Fig. 80, 81).

B) Midgut epithelium after *B. thuringiensis* treatment

The changes in the larval midgut epithelium in *Parnara guttata* were detected in one hour, two hours and four hours after intoxication of *B. thuringiensis* spore-crystal mixture.

i) One hour post-treatment

At the first hour after intoxication, the midgut epithelium seemed to be slightly affected. Increase in vacuolation was observed in the columnar cell, and the nucleus in these cells were found translocated to the basal region. The brush broder, on the other hand, seemed not be severely affected (Fig. 82). Histochemically, a layer of P.A.S. positive materials lined the surface of epithelium (Fig. 83). The protein level was high and the basic protein was abundant (Fig. 84, 85).

ii) Two hours post-treatment

As time past, the vacuoles in the columnar cells associated to form a medium size vacuole in the columnar cell, the nucleus of these vacuolated cells translocated to the base of the cell. The affected cells expanded and pushed the unaffected cells together (Fig. 86, 87). The basic protein level in midgut epithelium increased (Fig. 88, 89) and the P.A.S. positive layer on the apices of columnar cells were not seriously affected (Fig. 90).

iii) Four hours post-treatment

Four hours after B. thuringiensis treatment, large vacuoles were formed in the columnar cells. The brush broder, however, seemed to be slightly affected. The nuclei of columnar cells were found near the basal region of the cells (Fig. 91, 92, 93). As the vacuoles further expanded, the cells lysed. Disintegration of the gut wall resulted and the larvae died later.

4.3) Recovery of bacteria from infected larvae

Under electron microscopy, the infected larvae were found contaminated with rod-shaped bacteria (Fig. 96, 97). Free spores were also observed in the midgut lumen of the infected larvae (Fig. 99, 99), and after extracting the whole midgut epithelium, a rod-shaped, gram-positive bacteria were found appeared only in the infected larval tissue extract (Fig. 94, 95). Therefore, this rod-shaped, gram-positive, spore-forming bacteria might be responsible for the toxicity in insect larvae. These bacteria were speculated to be the Bacillus thuringiensis.

Fig. 1 Adult Pieris canidia, top view, feeding on nectar of cabbage flower (1.5 X).

Fig. 2 Adult Pieris canidia, top view, feeding on nectar of cabbage flower. Note the black spots on the fore-wing and margin of both wings (1 X).


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Fig. 3 Larva of Pieris canidia at the fourth instar (arrowed), feeding on a leaf of a cabbage plant. Note the green colour of the caterpillar, the plant was seriously damaged. (1 X)


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Fig. 4 Gut of Pieris canidia larvae, diagramatic drawing. (x10) (col, colon; cr, crop; fg; foregut; hg, hindgut; il, ileum; midg, midgut; mt, malpighian tubule; oe, oesophagus; rec, rectum)

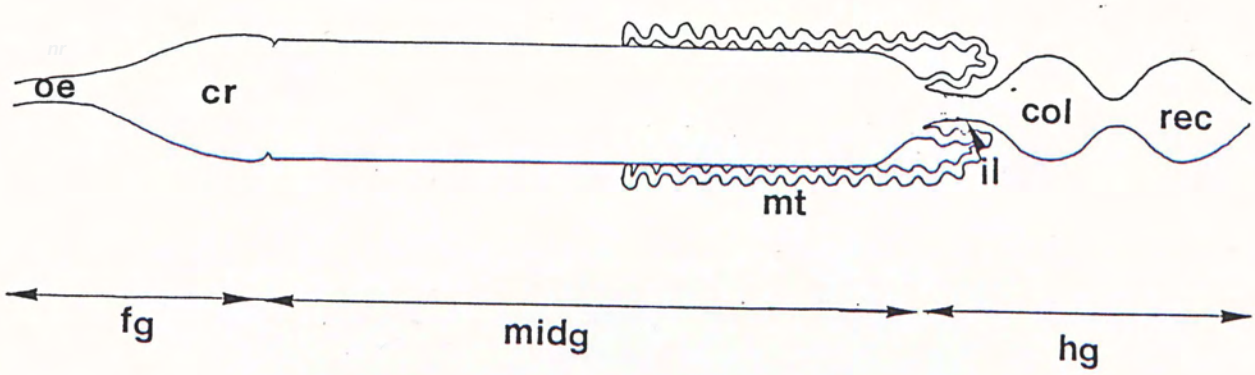

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Fig. 5 Structure of the midgut epithelium of Pieris canidia larvae, diagrammatic drawing. (bb, brush border; bm, basement membrane; c, columnar cell; cm, circular muscle; es, extracellular space;g, goblet cell; gc, goblet cavity; is, intercellular space; lm, longitudinal muscle; mi, mitochondria; mv, microvilli; n1,n2,n3, nucleus; pm, peritrophic membrane; rc, regenerative cell; rer, rough endoplasmic reticulum.)

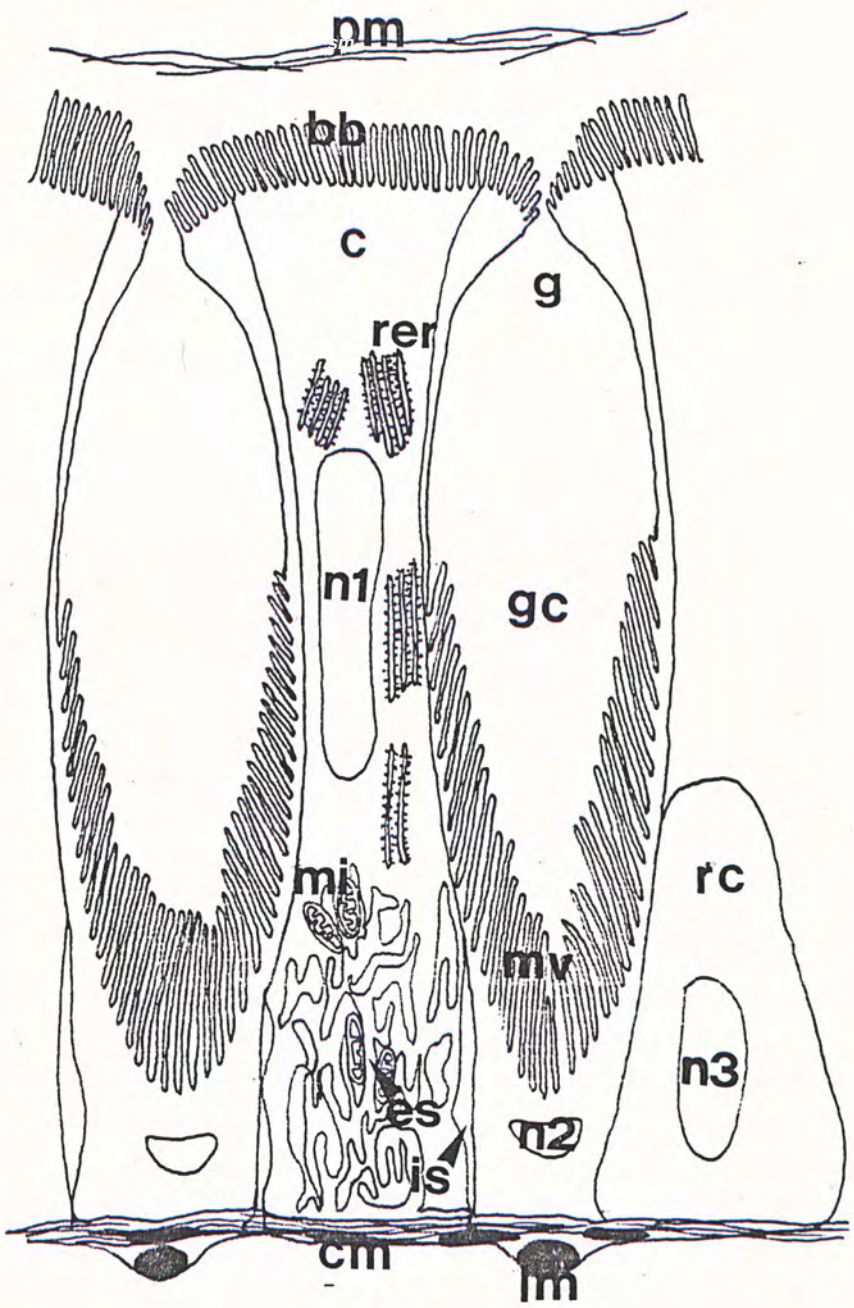

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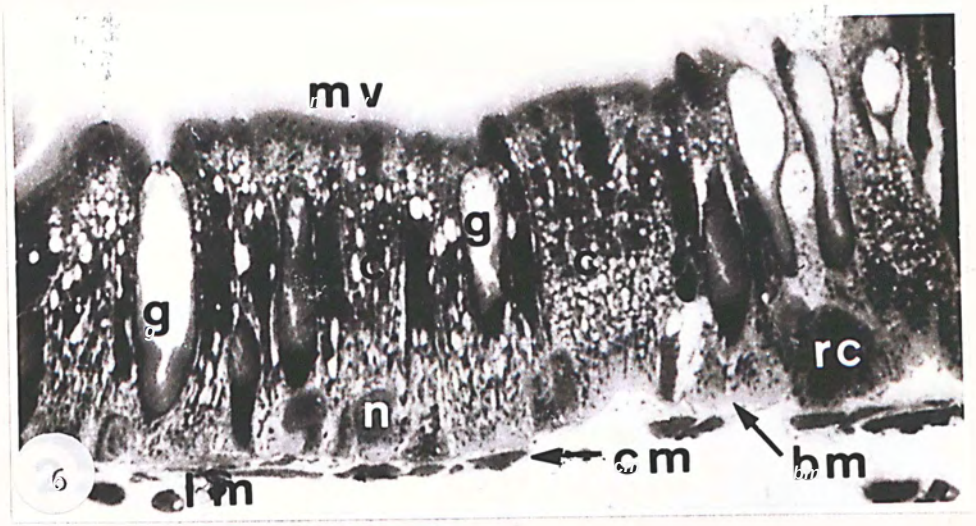
Fig. 6 Transverse section of the midgut epithelium of the fifth instar larva (*Pieris canidia*), without *B. thuringiensis* treatment. (Toluidine blue stained x 400, thick section) (bm, basement membrane; c, columnar cell; cm, circular muscle; g, goblet cell; lm, longitudinal muscle; mv, microvilli; n, nucleus; rc, regenerative cell.)


mv
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g
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n
cm
bm
lm

Fig. 7 Apical region of a columnar cell in the midgut epithelium of fifth instar larva (Pieris canidia). (TEM x 8280) Note the rough endoplasmic reticulum, mitochondria, and vacuole. (mi, mitochondria; mv, microvilli; rer, rough endoplasmic reticulum; v, vacuole.)

Fig. 8 Central region of a columnar cell in the midgut epithelium of fifth instar larva (Pieris canidia). (TEM x 3240) Note the centrally located nucleus, the arrangement of rough endoplasmic reticulum, the mitochondria, the intercellular space and extracellular space of the columnar cell. (es, extracellular space; is, intercellular space; mi, mitochondria; n, nucleus; rer, rough endoplasmic reticulum.)


mv
rer
v
mi
7

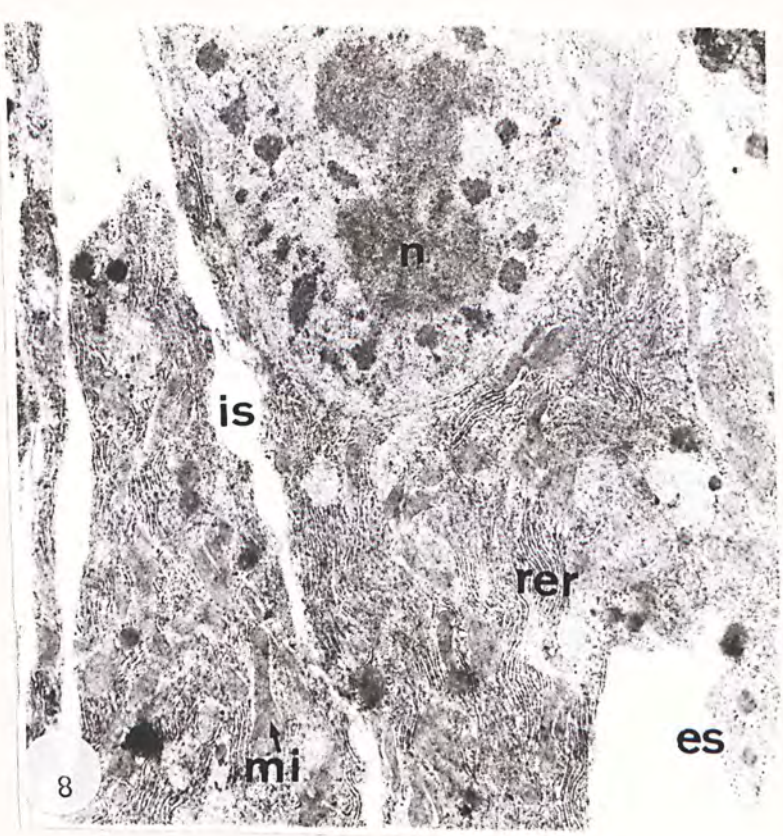
n
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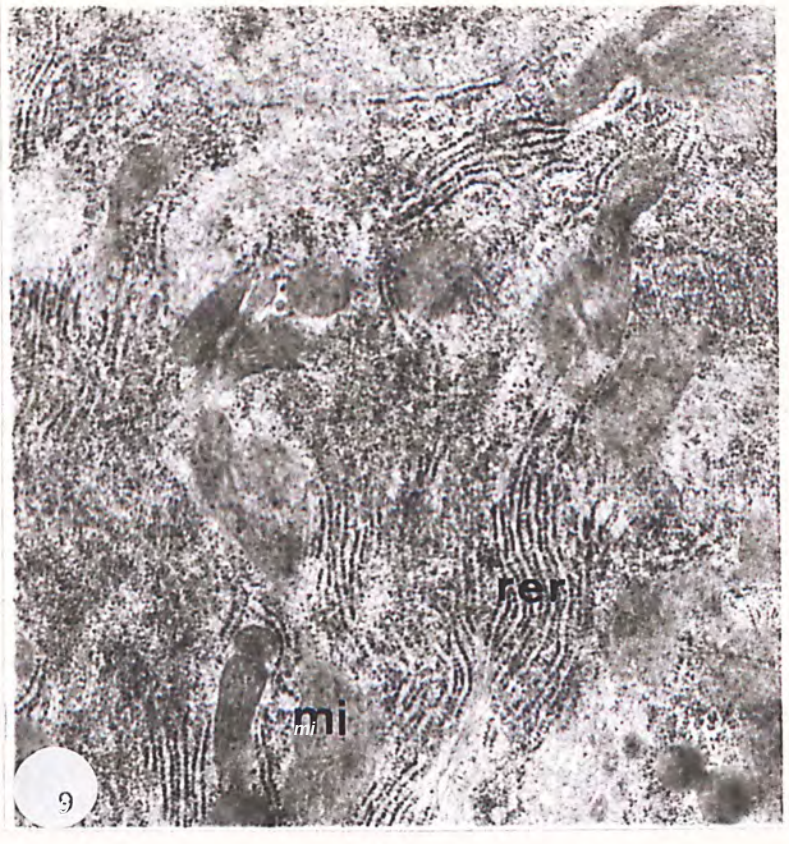
Fig. 9 Columnar cell from the midgut epithelum of the fifth instar larva of Pieris canidia. (TEM x 8280) Note the rough endoplasmic reticulum and mitochondria. (mi, mitochondria; rer, endoplasmic reticulum)

Fig. 10 Goblet cell from the midgut epithelium of the fifth instar larva of Pieris canidia, (TEM x 5400). Note the elongated mitochondria carried in the microvilli of a goblet cell. (gc, goblet cavity; mi, mitochondria; mv, microvilli.)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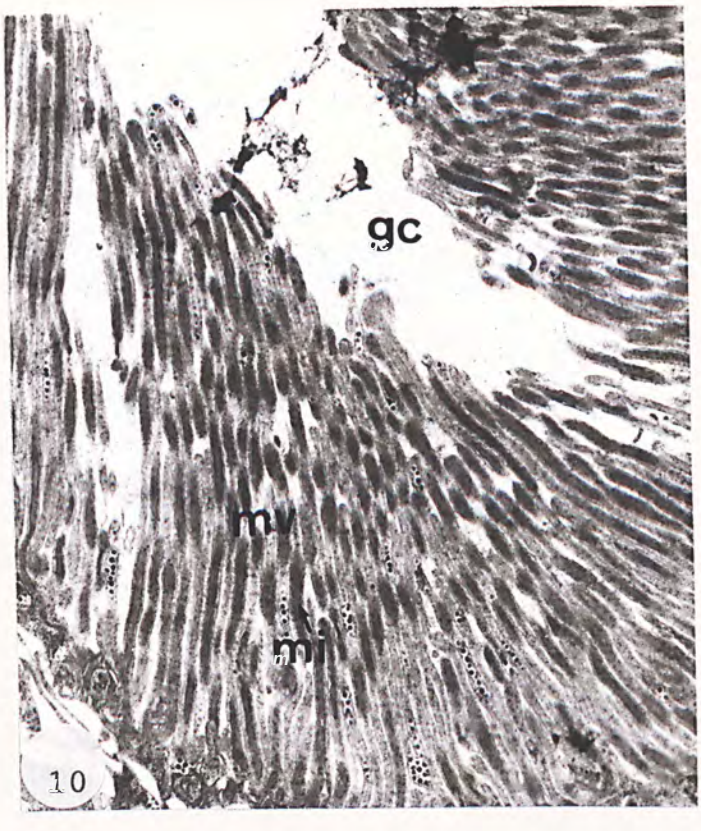
gc
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mi
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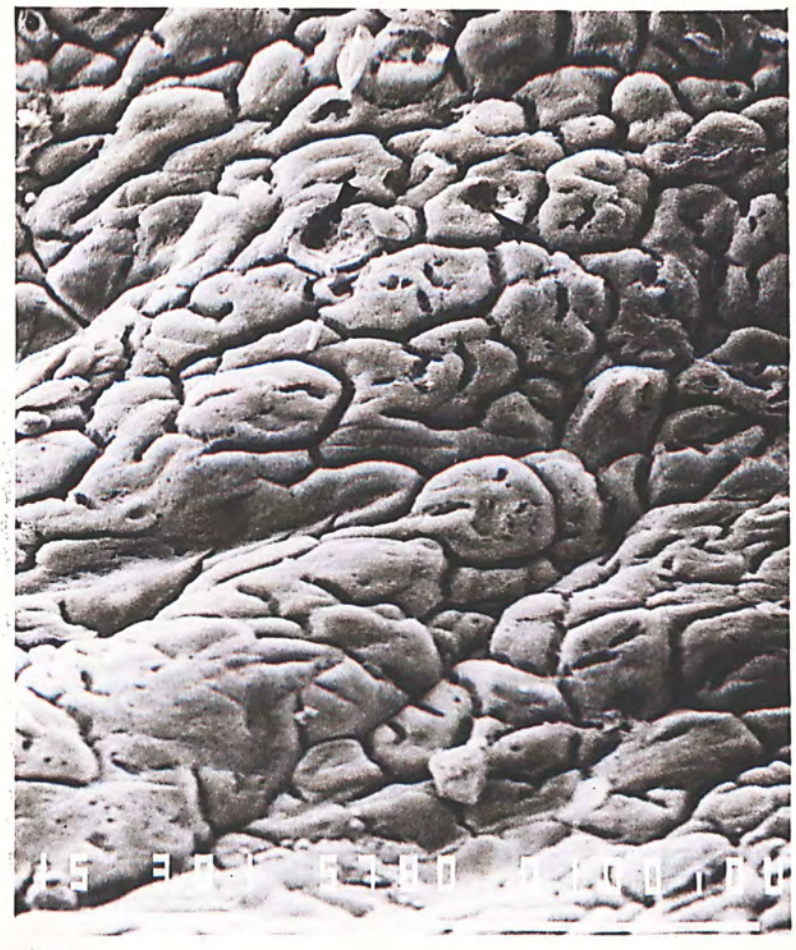
Fig. 11 The midgut epithelium of the fifth instar larvae of Pieris canidia showing the luminal side of the gut. (SEM x 450) Groups of columnar cells appear as crests. Position of an arrow head points to an orifice of a goblet cell.

Fig. 12 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), one hour after B. thuringiensis treatment. (Toluidine blue stained x 400, thick resin section) The columnar cell was found swollen, increased in vacuolation and secretion. Intercellullar spaces increased in size between adjacent columnar cells. Normal goblet cells were found in the epithelium. Single cells of B. thuringiensis were found penetrating into a columnar cell. (bm, basement membrane; bt, B. thuringiensis single cell; c, columnar cell; g, goblet cell; is, intercellular space; pm, peritrophic membrane; rc, regenerative cell; s, secretory materials; v, vacuole.)



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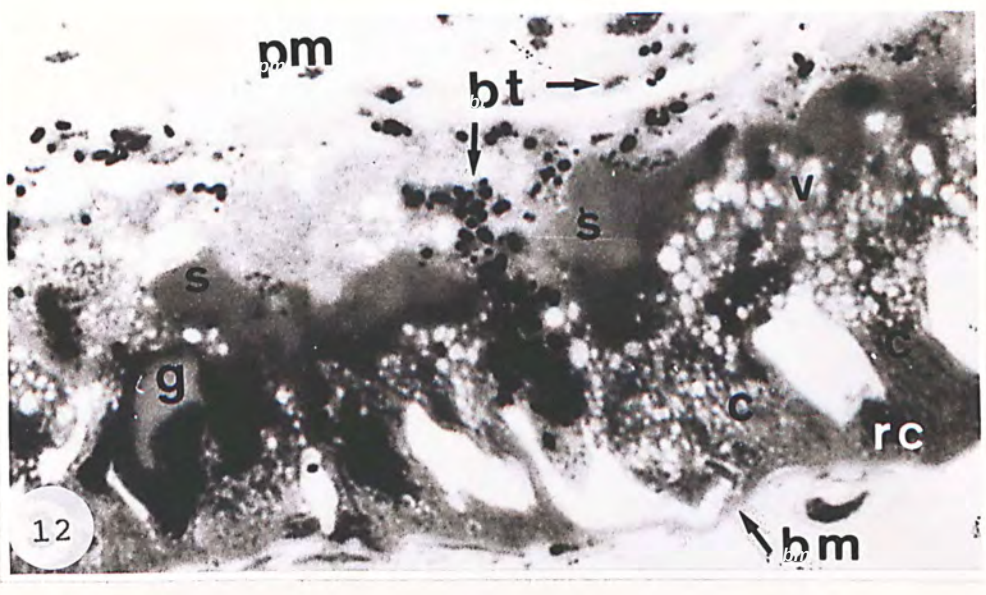

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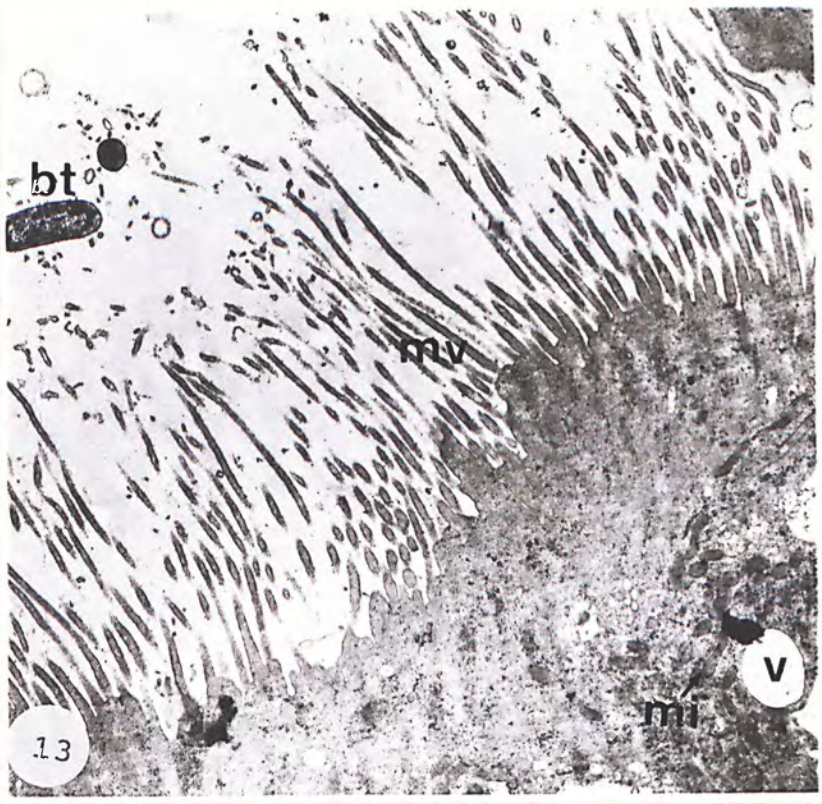
Fig. 13 Columnar cell from the midgut epithelium of the fifth instar of Pieris canidia one hour after B. thuringiensis treatment. (TEM x 3240) Note the disruption of microvilli in a columnar cell. (bt, B. thuringiensis; mi, mitochondria; mv, microvilli; v, vacuole)

Fig. 14 Columnar cell from the midgut epithelium of the fifth instar of Pieris canidia one hour after B. thuringiensis treatment. (TEM x 3240) Note the centrally located nucleus, and the inflated intercellular space and extracellular space of a columnar cell. (es, extracellular space; is, intercellular space; n, nucleus)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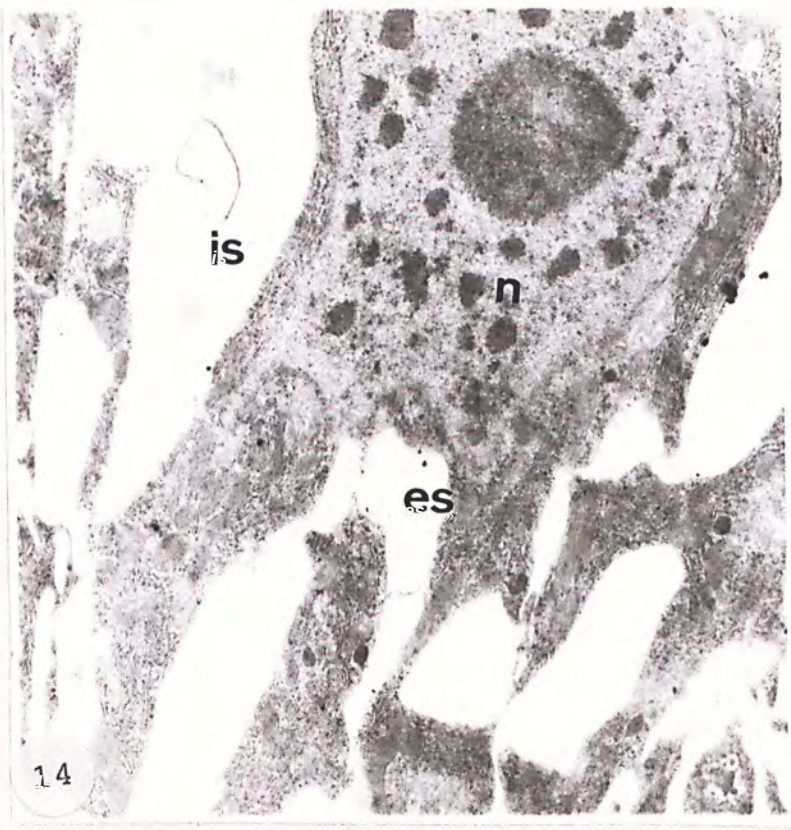
is
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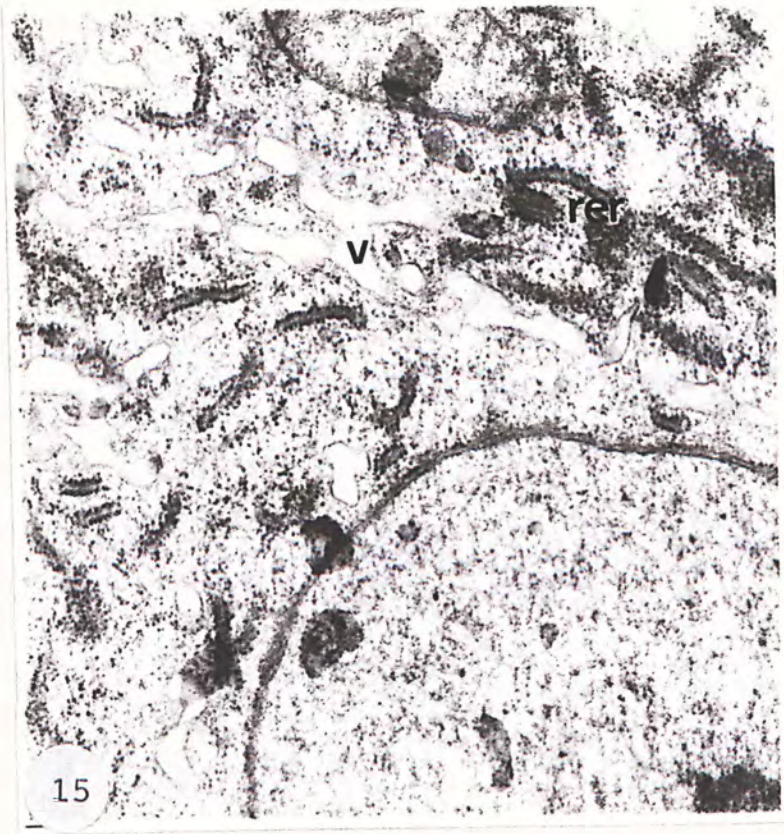
Fig. 15 Columnar cell from the midgut epithelium of the fifth instar of Pieris canidia one hour after B. thuringiensis treatment. (TEM x 17100) Note the disruption of rough endoplasmic reticulum and increase in vacuoles in the cytoplasm. (rer, rough endoplasmic reticulum; v, vacuole)

Fig. 16 Columnar cell from the midgut epithelium of the fifth instar of Pieris canidia one hour after B. thuringiensis treatment. (TEM x 17100) Note the mitochondria in the cytoplasm. (mi, mitochondria)


rer
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mi
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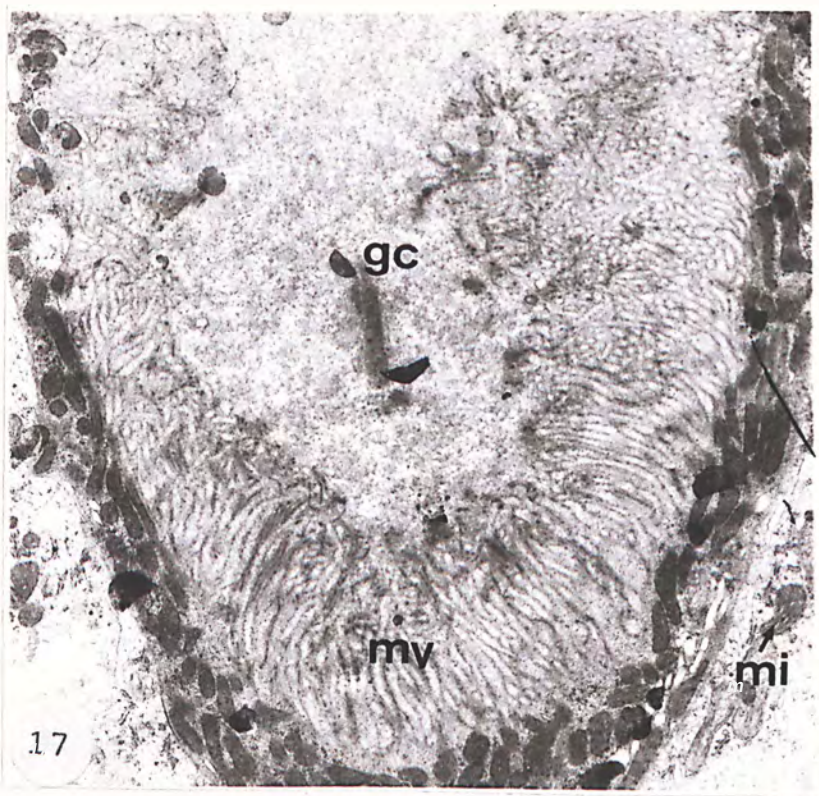
Fig. 17 The goblet cell from the midgut epithelium of the fifth instar of Pieris canidia one hour after B. thuringiensis treatment. (TEM x 3240) Note the goblet cavity, the microvilli, and the mitochondria. (gc, goblet cavity; mi, mitochondria; mv, microvilli)

Fig. 18 The midgut epithelium of the fifth instar of Pieris canidia one hour after B. thuringiensis treatment, showing the luminal side of the midgut epithelium. (SEM x 450) Note that the surface started disintegrating.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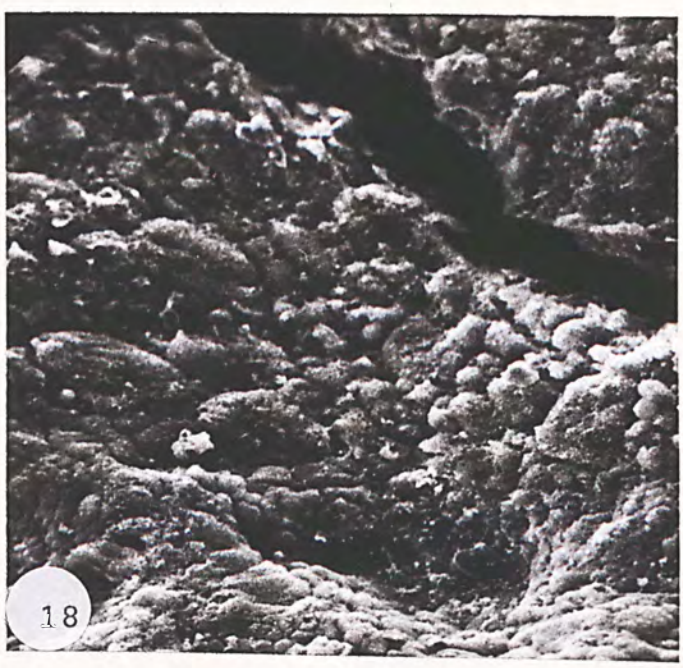
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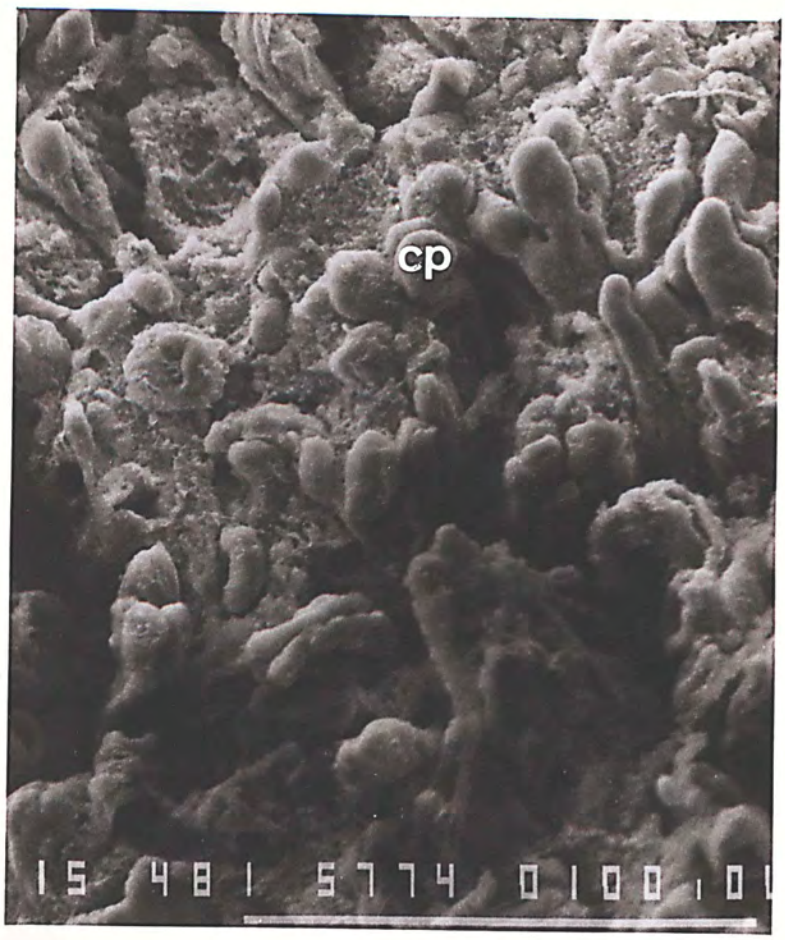
Fig. 19 Apical cytoplasmic projection on the surface of midgut epithelium of the fifth instar larva of Pieris canidia one hour after B. thuringiensis treatment. (SEM x 864) (cp, cytoplasmic projection)

Fig. 20 Transverse section of the midgut epitheliumof fifth instar larva (Pieris canidia), two hours after B. thuringiensis treatment. (Toluidine blue stained x 400, thick resin section) The columnar cell was found swollen, increased in vacuolation and secretion. On its apical region, disruption of brush border, and appearance of cytoplasmic projections were observed. Infoldings of basal cytoplasmic membrane extended to 2/3 length of the columnar cell. Single cells of B. thuringiensis were found penetrating into a columnar cell. (bt, B. thuringiensis single cell; c, columnar cell; cp, cytoplasmic projection; mv, microvilli; rc, regenerative cell; s, secretory materials)



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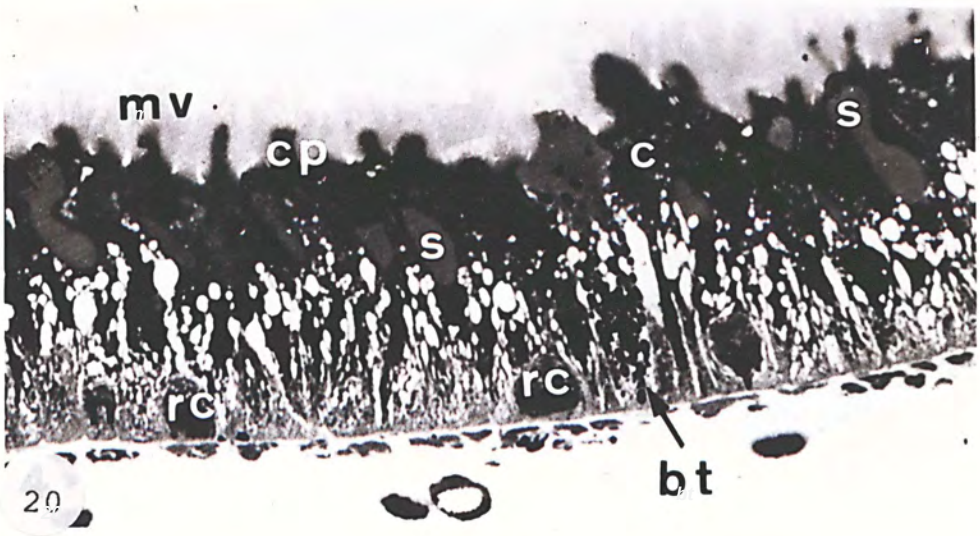

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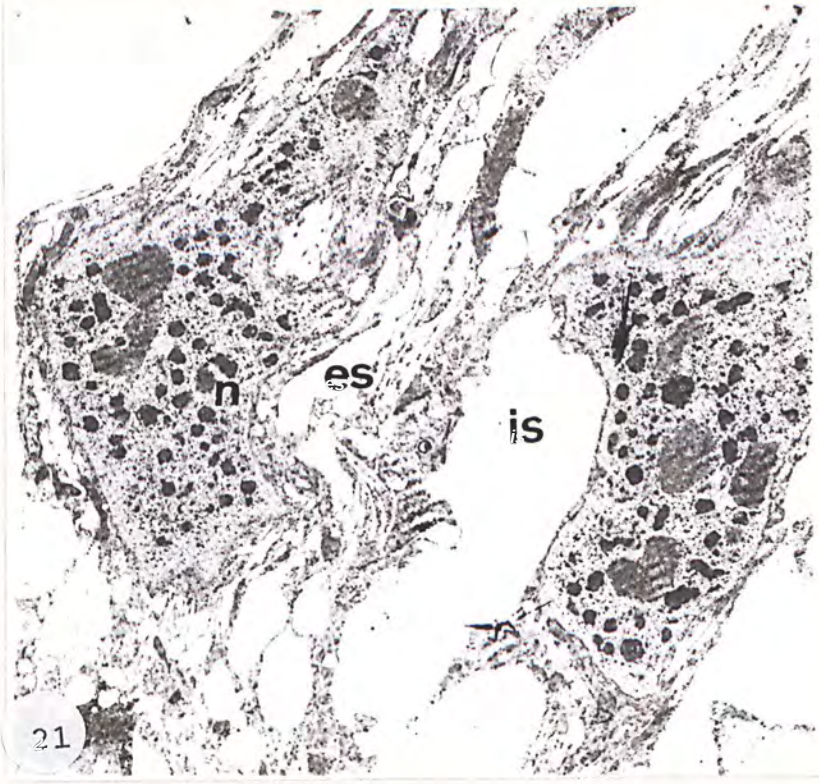
Fig. 21 The columnar cells from the midgut epithelium of the fifth instar of Pieris canidia two hours after B. thuringiensis treatment. (TEM x 3240) Notethe increase of intercellular space and extracelllular space. (es, extracellular space; is, intercellular space; n, nucleus)

Fig. 22 The columnar cells from the midgut epithelium of the fifth instar of Pieris canidia two hours after B. thuringiensis treatment. (TEM x 1440) Note the increase of intercellular space and the extension of extracellular space result in the increase of infoldings of the basement membrane in the columnar cell. (es, extracellular space; is, intercellular space; n, nucleus)


n
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is
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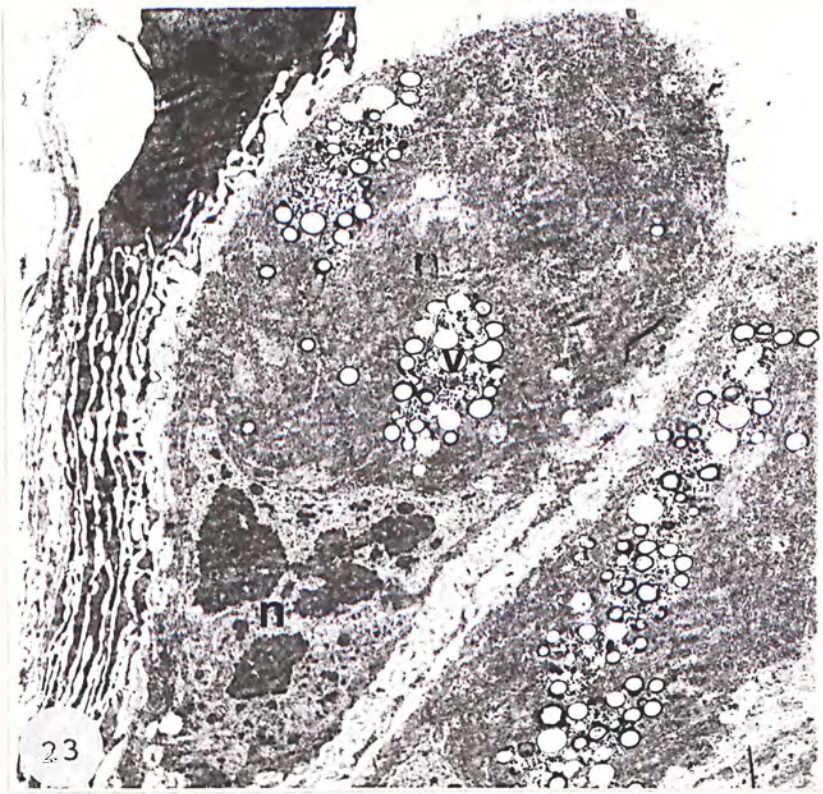
es
22

Fig. 23 The columnar cells from the midgut epithelium of the fifth instar of Pieris canidia two hours after B. thuringiensis treatment. (TEM x 3240) Note the damaged columnar cell, heavy vacuolation was found in the cytoplasm. (n, nucleus; v, vacuole)

Fig. 24 The midgut epithelium of the fifth instar of Pieris canidia two hours after B. thuringiensis treatment, showing the luminal side of the midgut epithelium. (SEM x 450) Note that the surface had disintegrated.



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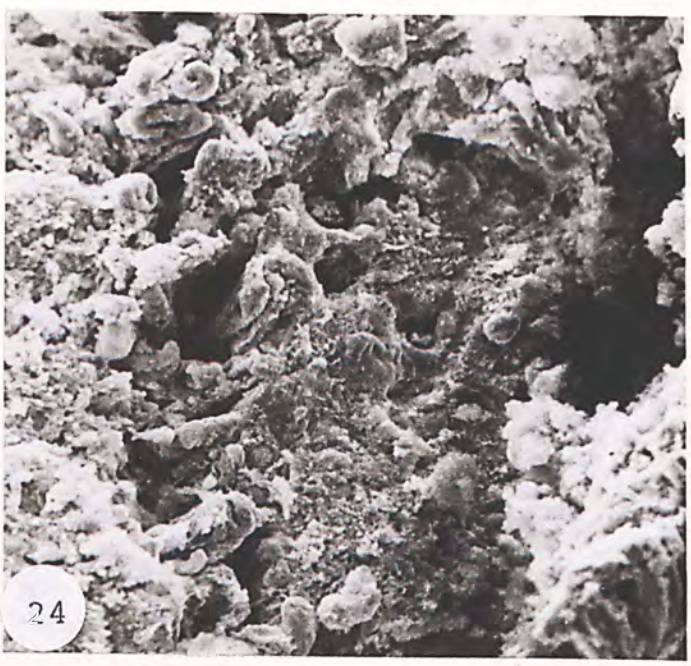

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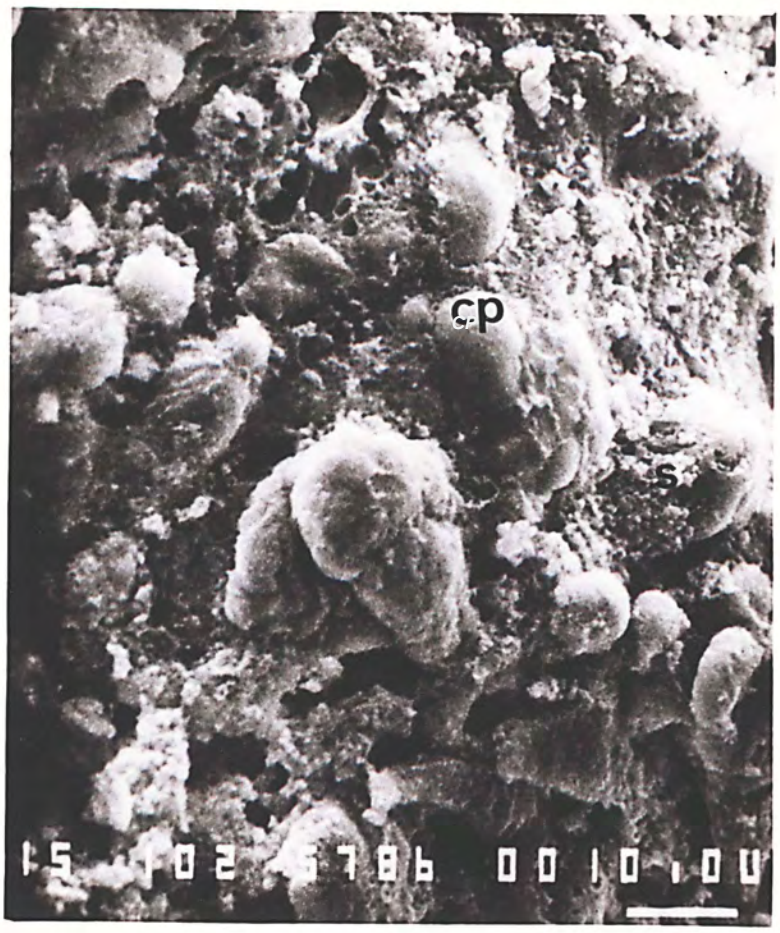
Fig. 25 The midgut epithelium of the fifth instar of Pieris canidia two hour after B. thuringiensis treatment, showing the luminal side of the midgut epithelium. (SEM x 1500) Note the secretous materials emerging from the midgut epithelium. (cp, cytoplasmic projection; s, secretory materials)

Fig. 26 The midgut epithelium of the fifth instar of Pieris canidia two hour after B. thuringiensis treatment, showing the luminal side of the gut wall. (SEM x 1800) Note the secretory materials emerged from the midgut epithelium. (cp, cytoplasmic projection; s, secretory materials)



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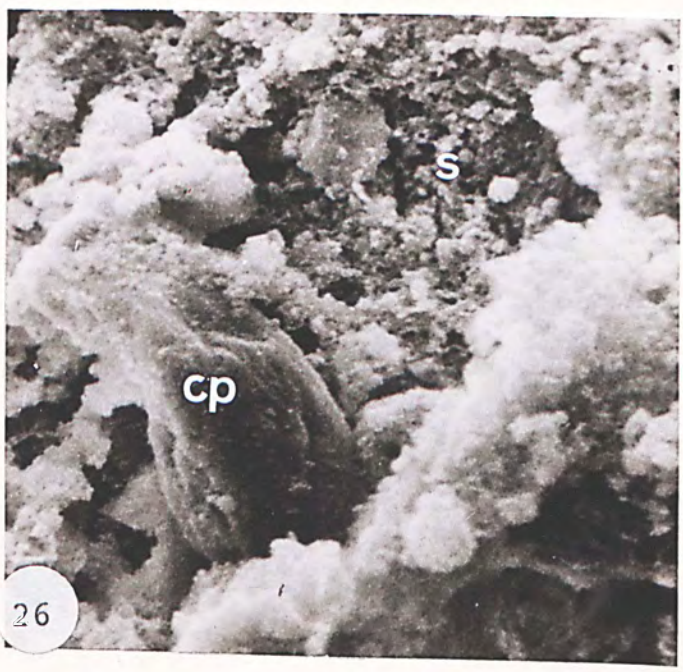

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Fig. 27 Transverse section of the midgut epitheliumof fifth instar larva (Pieris canidia) three hours after B. thuringiensis treatment. (Toluidine blue stained x 400, thick resin section) The columnar cell was found swollen, and extrusion of cytoplasm was observed. Some of the columnar cells lysed and space was left in the midgut epithelium. Single cells of B. thuringiensis were found penetrating into a columnar cell. (bm, basement; bt, B. thuringiensis single cell; c, columnar cell; cm, circular muscle; cp, cytoplasmic projection; g, goblet cell; rc, regenerative cell; lm, longitudinal muscle; lu, lumen; n, nucleus; rc, regenerative cell, sp, space;)

Fig. 28 Transverse section of the midgut epitheliumof fifth instar larva (Pieris canidia), four hours after B. thuringiensis treatment. (Toluidine blue stained x 400, thick resin section) The columnar cell was found swollen, and extrusion of cytoplasm was observed. Some of the columnar cells lysed and space was left on the midgut epithelium. The goblet cells swelled and sloughed off from the midgut epithelium. Single cell of B. thuringiensis was found in the infected cells. (bt, B. thuringiensis cell; c, columnar cell; cp, cytoplasmic projection; ec, extruded cytoplasm; fb, fat body; g, goblet cell; rc, regenerative cell)

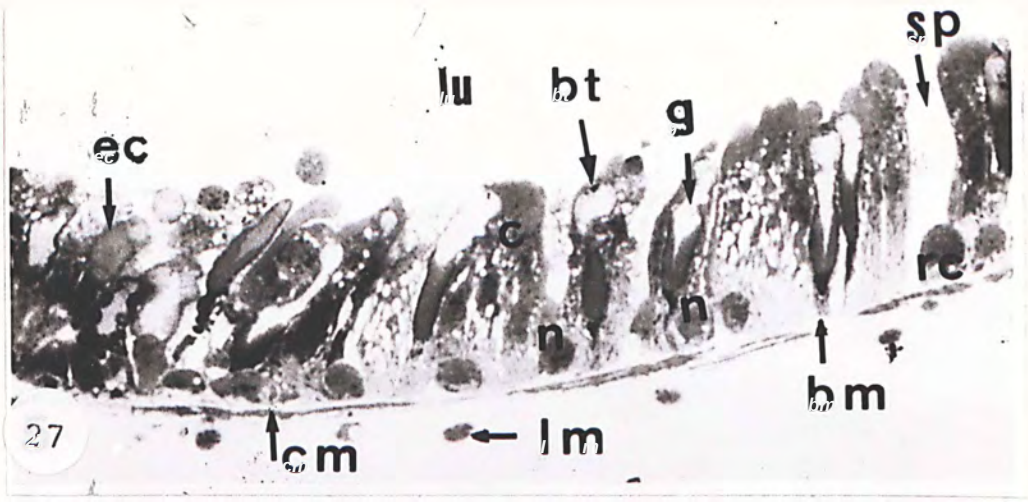

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Fig. 29 The midgut epithelium of the fifth instar of Pieris canidia four hour after B. thuringiensis treatment, showing the luminal side of the midgut epithelium. (SEM x 450) Note the disintegrated surface, numerous secretory bodies and the cytoplasmic projections emerging from the midgut epithelium. (cp, cytoplasmic projection; s, secretory materials)

Fig. 30 The midgut epithelium of the fifth instar of Pieris canidia four hour after B. thuringiensis treatment, showing the luminal side of the gut wall. (SEM x 1080) Note the disintegrated surface, numerous secretory bodies and the cytoplasmic projections emerging from the midgut epithelium and holes were left on the midgut epithelium (cp, cytoplasmic projection; s, secretory materials)

29

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Fig. 31 Transverse section of the midgut epithelium of fifth instar larva (*Pieris canidia*), five hours after *B. thuringiensis* treatment. (Toluidine blue stained x 400, thick resin section). Most of the epithelial cells sloughed off the midgut epithelium, the residual cell materials were heavily contaminated by the bacteria. (bt, *B. thuringiensis* cells; bm, basement membrane; cm, circular muscle; lm, longitudinal muscle; rcm, residual cell materials; tr, tracheole)

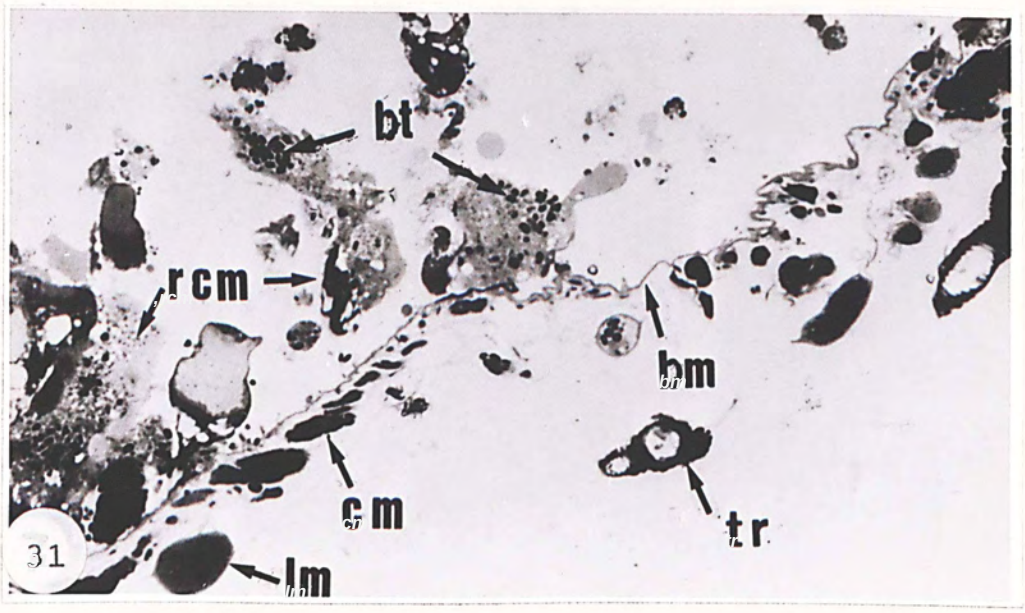

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Fig. 32 Transverse section of the midgut epithelium of fifth instar larva (*Pieris canidia*), without *B. thuringiensis* treatment. (Mercury-Bromophenol blue stained x 400) The protein constituents were stained blue to brown. (c, columnar cell; g, goblet cell)

Fig. 33 Transverse section of the midgut epithelium of fifth instar larva (*Pieris canidia*), without *B. thuringiensis* treatment. (Acid solochrome cyanine stained x 400) Basic protein was stained red in this method. Note the epithelium was not heavily stained, espically on the brush border and the secretory materials in the goblet cavity. (bb, brush border; g, goblet cell; s, secretory materials.)

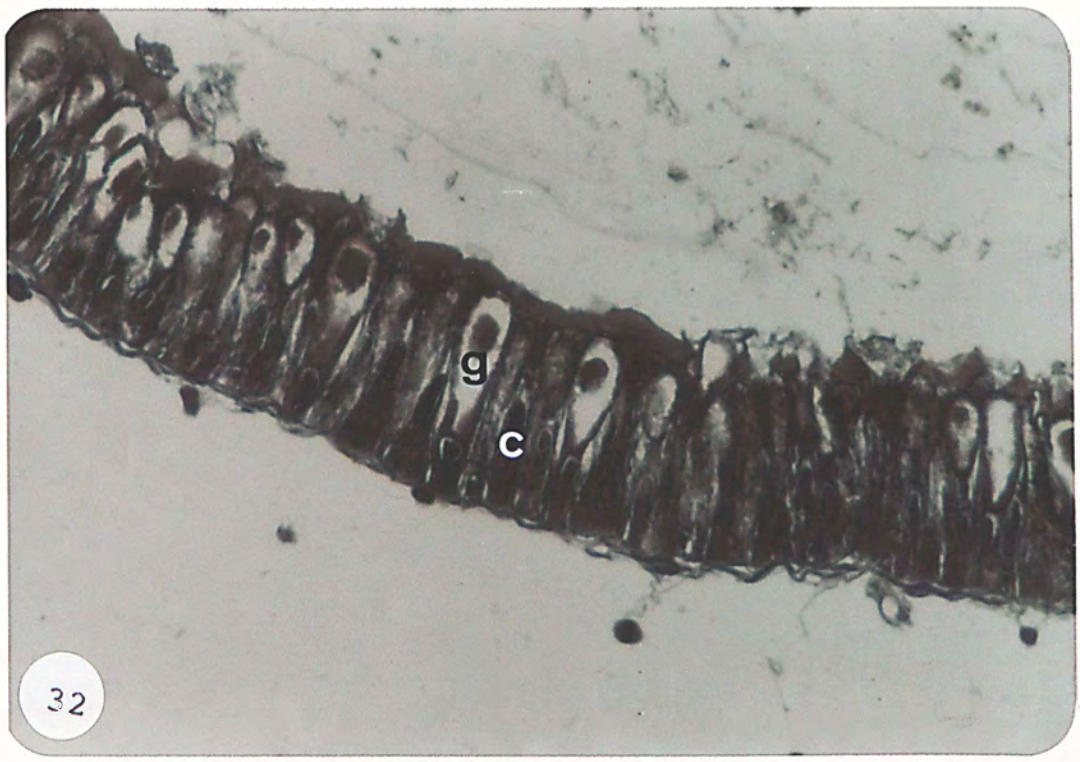
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Fig. 34 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), without B. thuringiensis treatment. (P.A.S. stained x 400) The brush border and secretory substance in the goblet cavities were stained purple-red (positive) owing to the presence of a mucous substance secreted, the muscle sheath was also positively stained. The cytoplasm, the nuclei of the columnar cell and the goblet cell were stained blue. (bb, brush border; c, columnar cell; g, goblet cell; m, muscle sheath; s, secretory materials)

Fig. 35 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), without B. thuringiensis treatment. (Alcian Blue stained x 400) method. The acid mucin and sulfide containing mucin were stained blue, no positive result was found on the epithelium. (c, columnar cell; g, goblet cell)

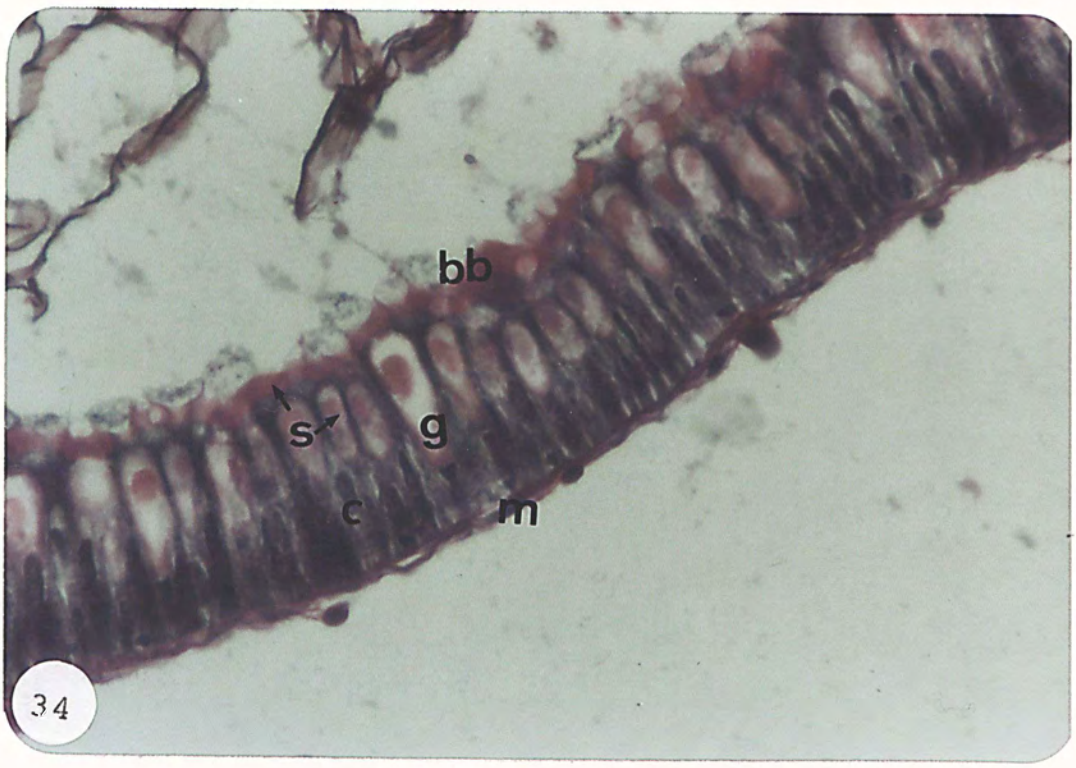
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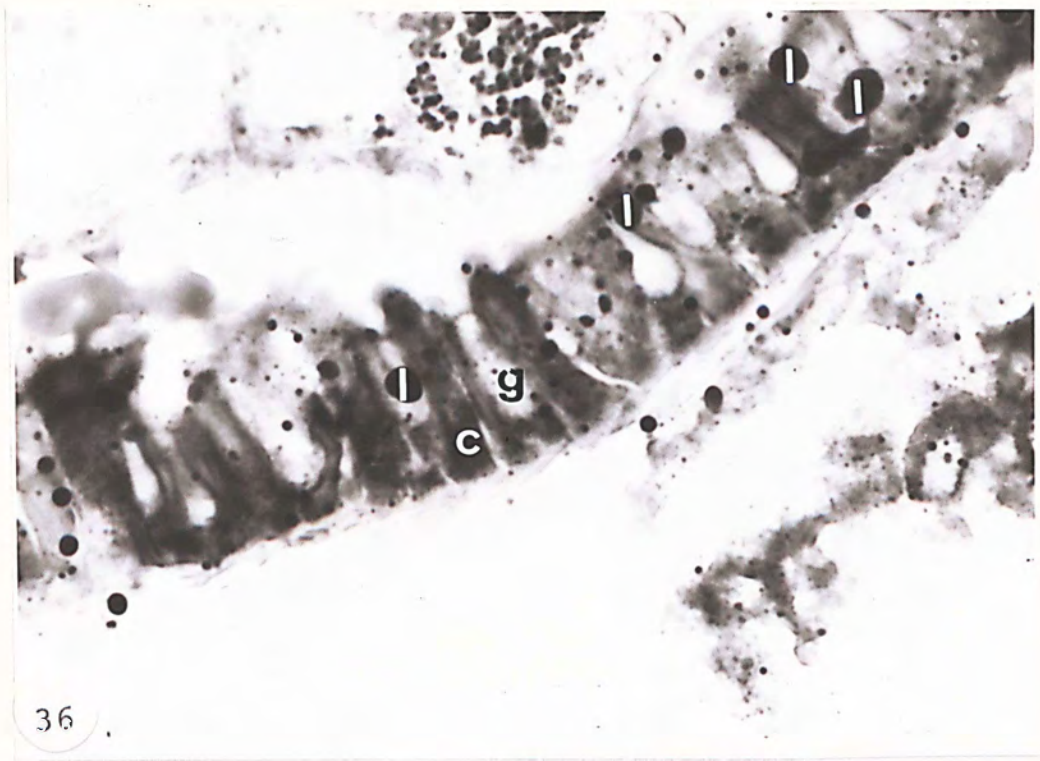
Fig. 36 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia), without B. thuringiensis treatment. (Sudan Black stained x 400) The lipids droplets were stained Black. (c, columnar cell; g, goblet cell; l, lipid droplet)

Fig. 37 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia), without B. thuringiensis treatment. (Substituted Naphthol stained x 400) The activity of alkaline phosphatase was demonstrated by precipitation of a red azo dye. Note the red colour was extensively located on the brush border. (bb, brush border; c, columnar cell; g, goblet cell)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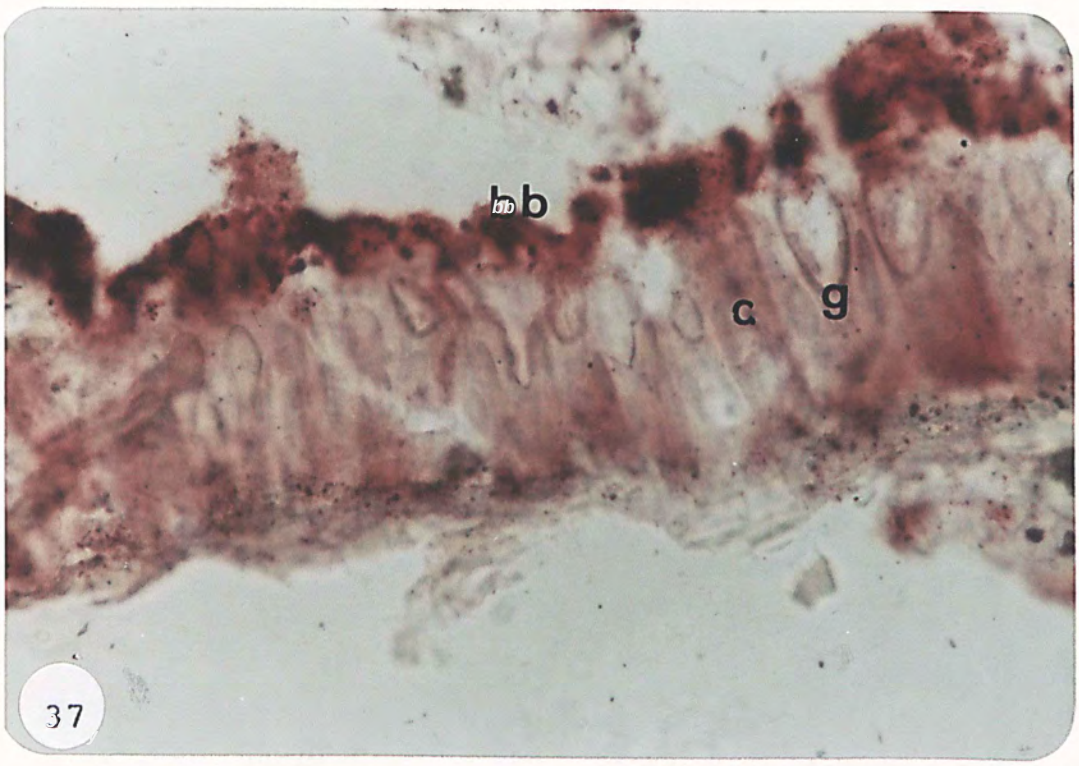
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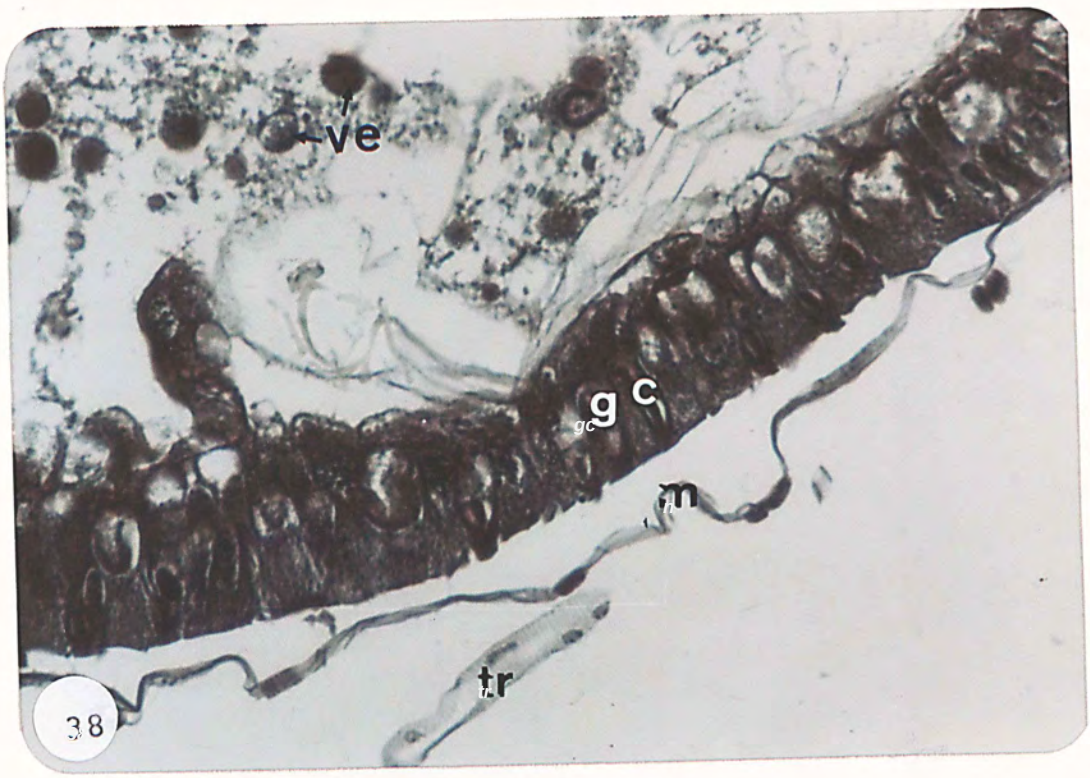
Fig. 38 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), 20 minutes after B. thuringiensis treatment. (Mercury-Bromophenol blue stained x 400) Protein constitutents were stained blue to brown Vesicles were extruded from the cytoplasm to the gut lumen. The muscle sheath and tracheole were also stained blue. (c, columnar cell; g, goblet cell; m, muscle sheath; tr, tracheole; ve, extruded vesicle)

Fig. 39 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), 20 minutes after B. thuringiensis treatment. (Acid solochrome cyanide stained x 400) Basic protein was stained red. Note the high level of basic protein in the epithelium. (c, columnar cell; cp, cytoplasmic projection; g, goblet cell; m, muscle sheath)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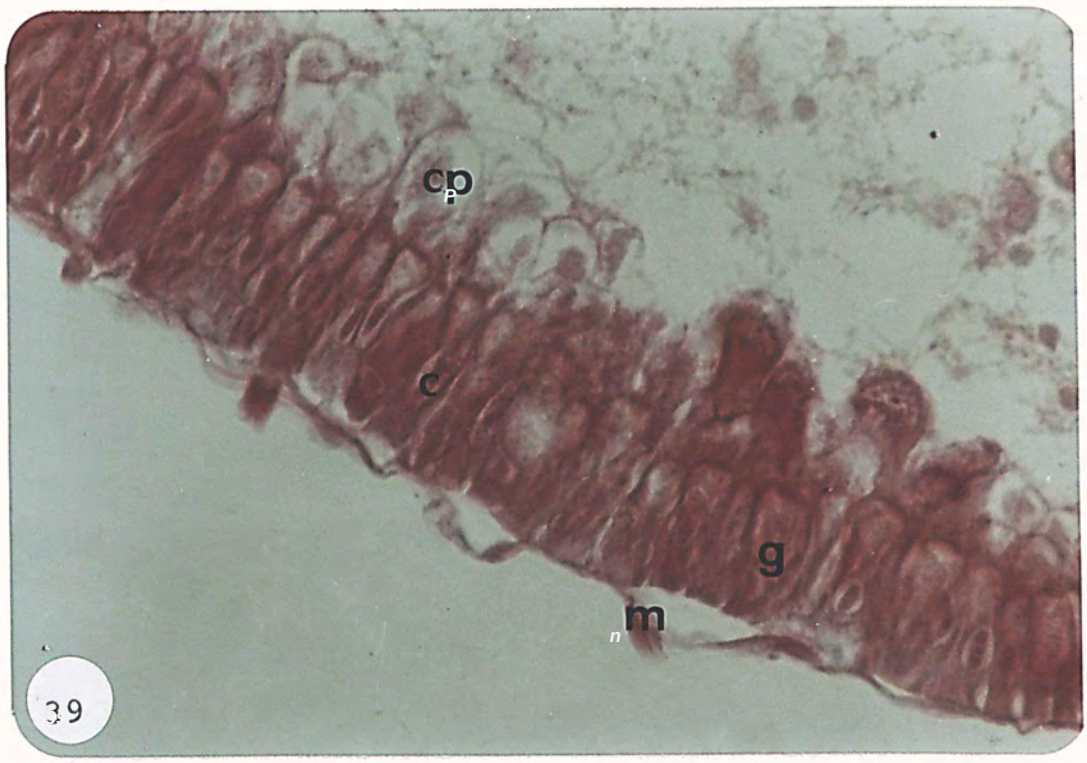
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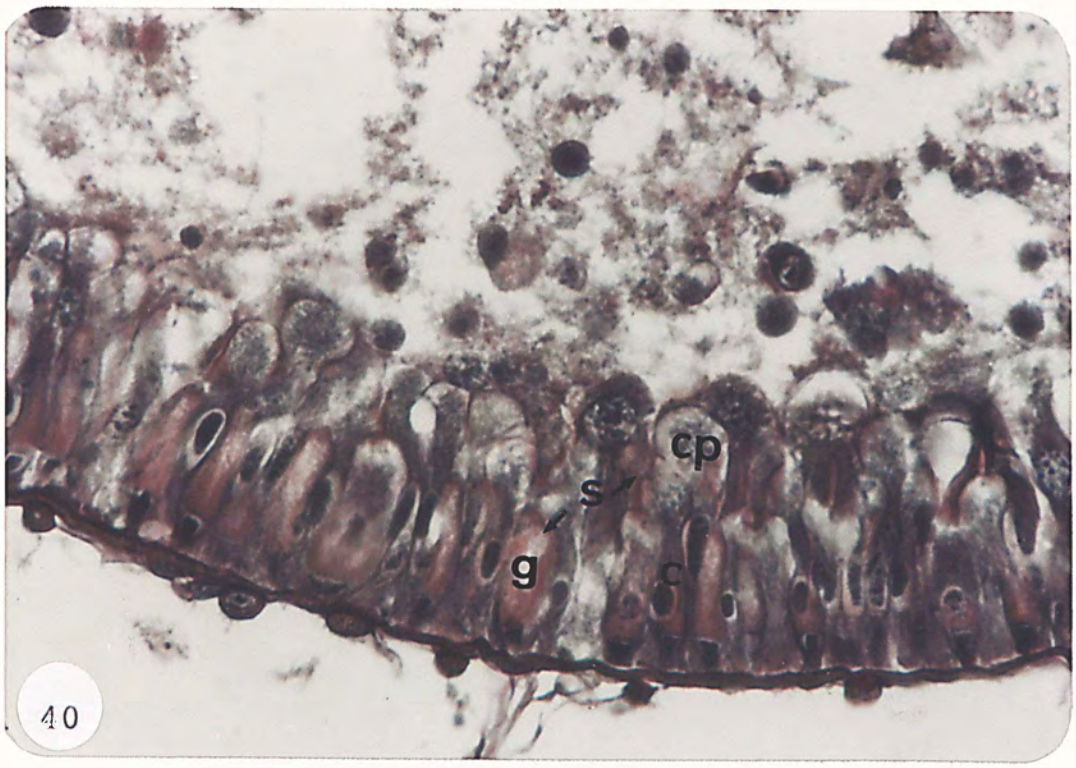
Fig. 40 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), 20 minutes after B. thuringiensis treatment. (P.A.S. stained x 400) The secretous materials were found on the surface of columnar cells, and goblet cavity which were stained purple-red (positive). The cytoplasm, the nuclei of the columnar cell and the goblet cell were stained blue. (c, columnar cell; cp, cytoplasmic projection; g, goblet cell; s, secretory material)

Fig. 41 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), 20 minutes after B. thuringiensis treatment. (Alcian Blue stained x 400) The acid mucin and sulfide containing mucin were stained blue, no positive result was found on the epithelium. The secretous substances in the goblet cavities were deeply stained by safranin (arrowed). (c, columnar cell; cp, cytoplasmic projection; g, goblet cell; m, muscle sheath; ve, extruded vesicle)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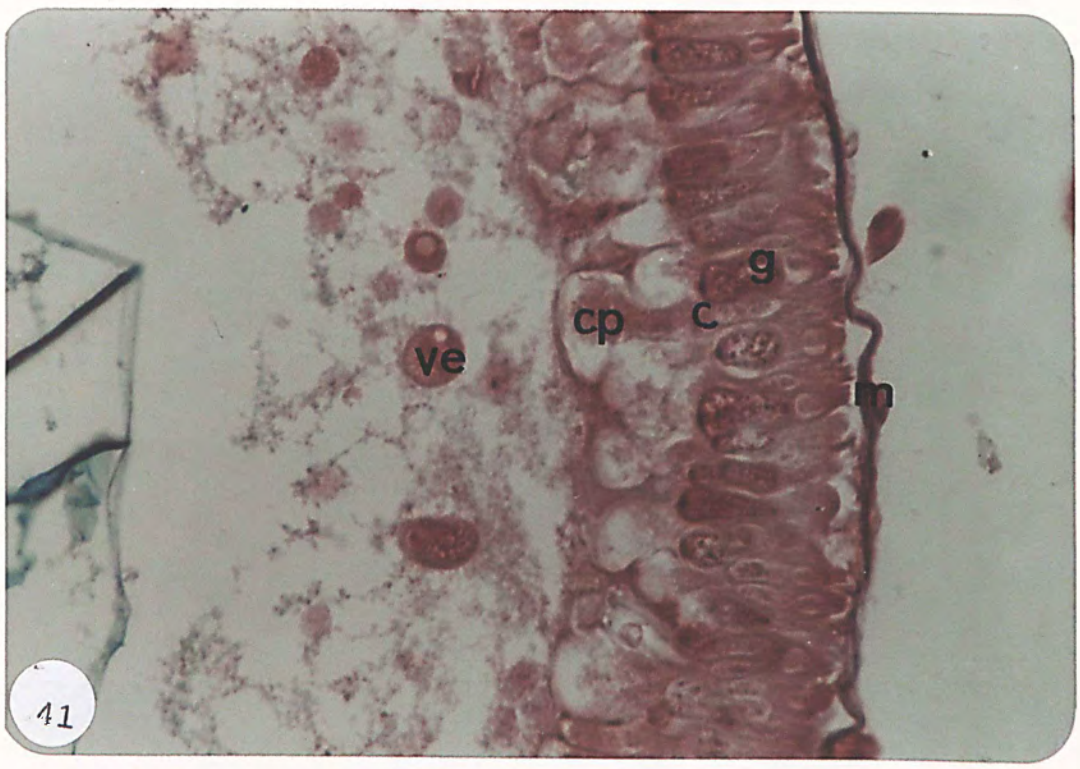
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m
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Fig. 42 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia), 20 minutes after B. thuringiensis treatment. (Sudan Black stained x 400) No lipids droplets could be detected. (c, columnar cell; g, goblet cell)

Fig. 43 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia), 20 minutes after B. thuringiensis treatment. (Substituted Naphthol stained x 400) The activity of alkaline phosphatase was demonstrated by precipitation of a red azo dye. Note the decline of red colour in the midgut epithelium. (c, columnar cell; g, goblet cell)

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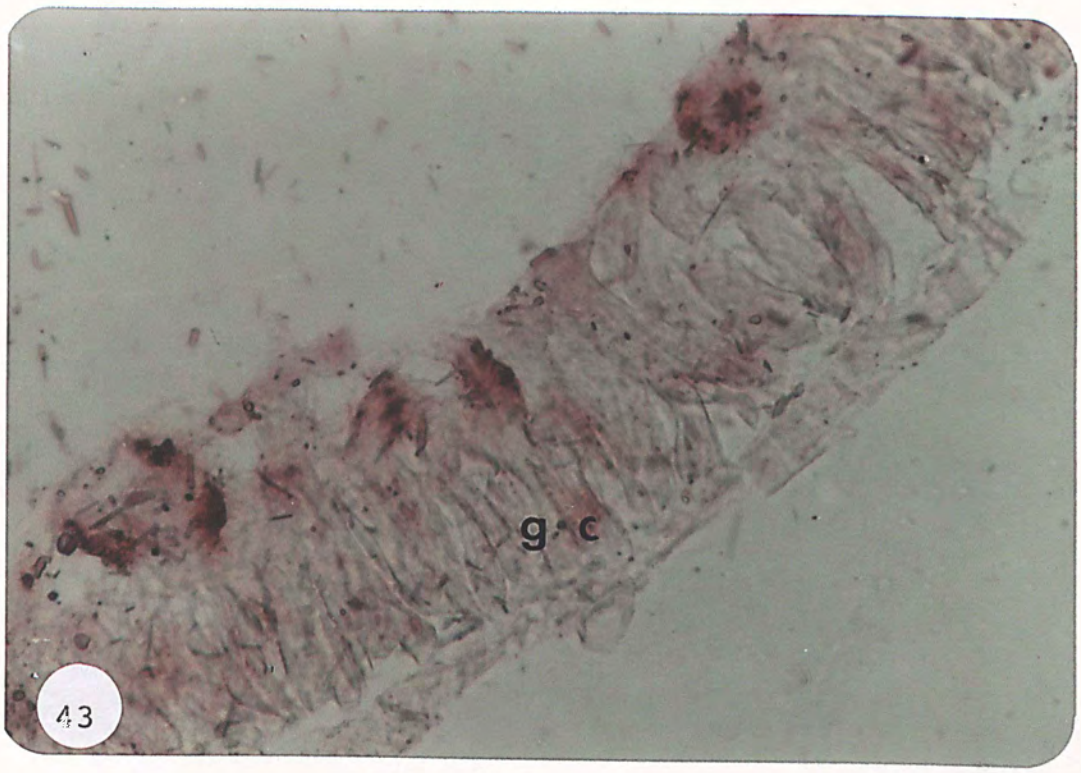

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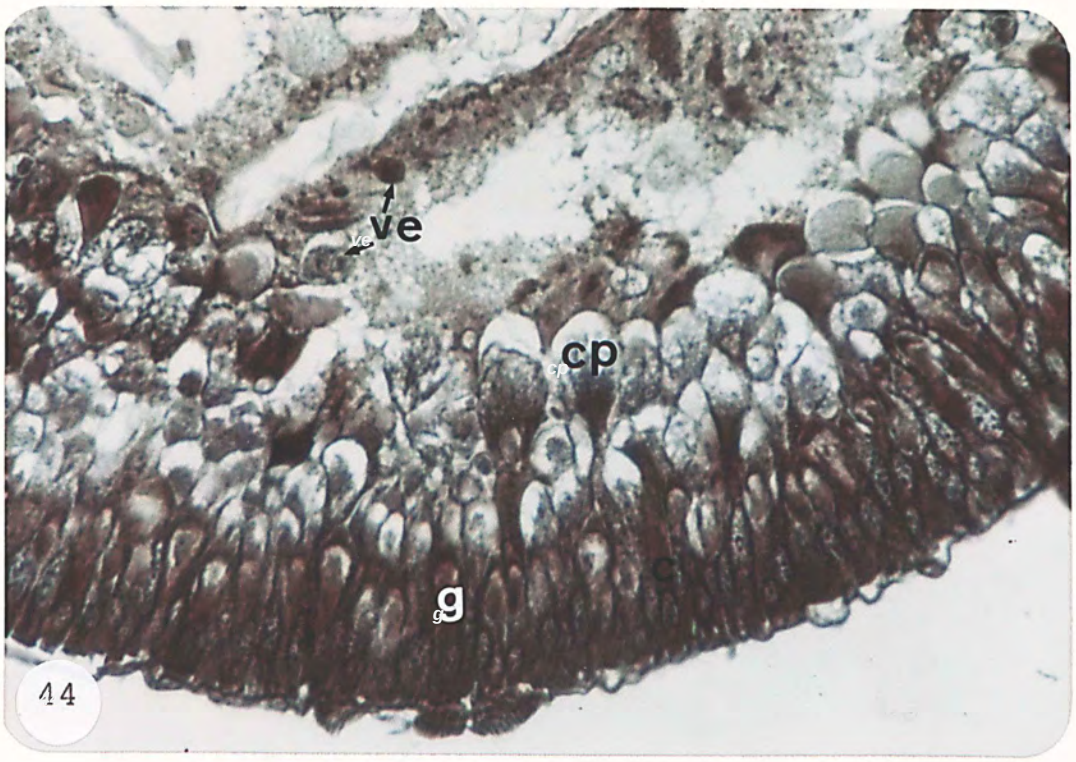
Fig. 44 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), 40 minutes after B. thuringiensis treatment. (Mercury-Bromophenol blue stained x 400) Protein constituents were stained blue to brown. Note extrusion of cytoplasm to the gut lumen. (c, columnar cell; cp, cytoplasmic projection; g, goblet cell; ve, extruded vesicle)

Fig. 45 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), 40 minutes after B. thuringiensis treatment. (Acid solochrome cyanine stained x 400) Note the high level of basic protein in the epithelium. (c, columnar cell; cp, cytoplasmic projection; g, goblet cell)



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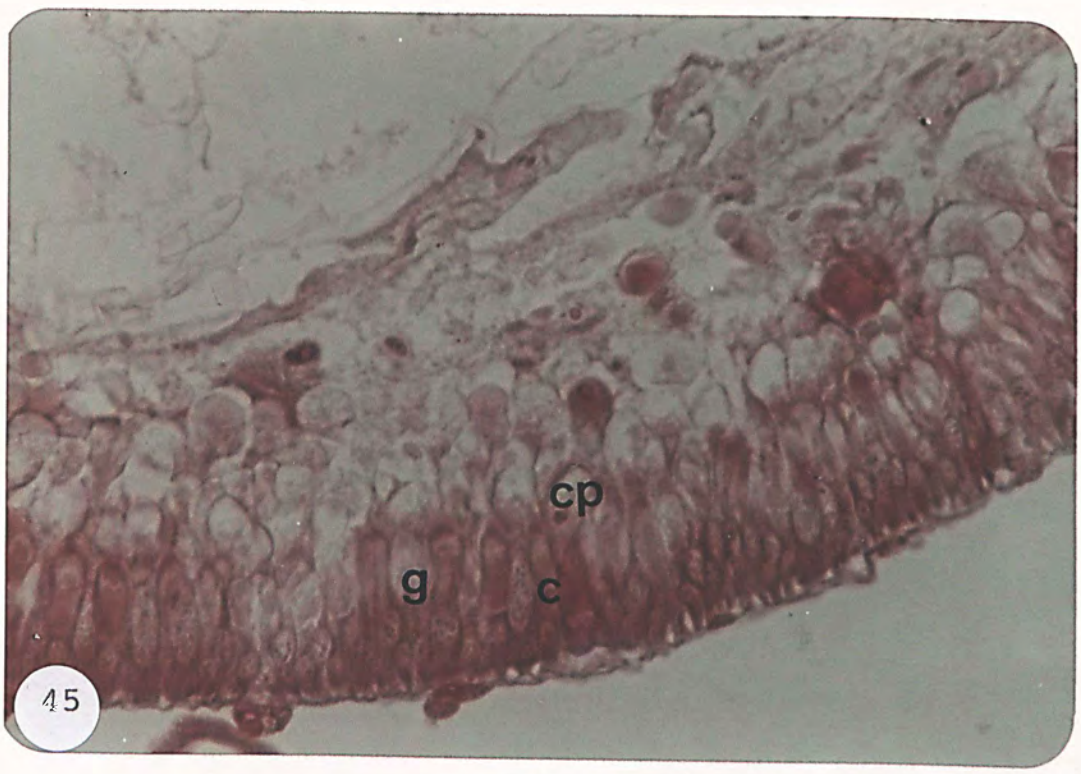

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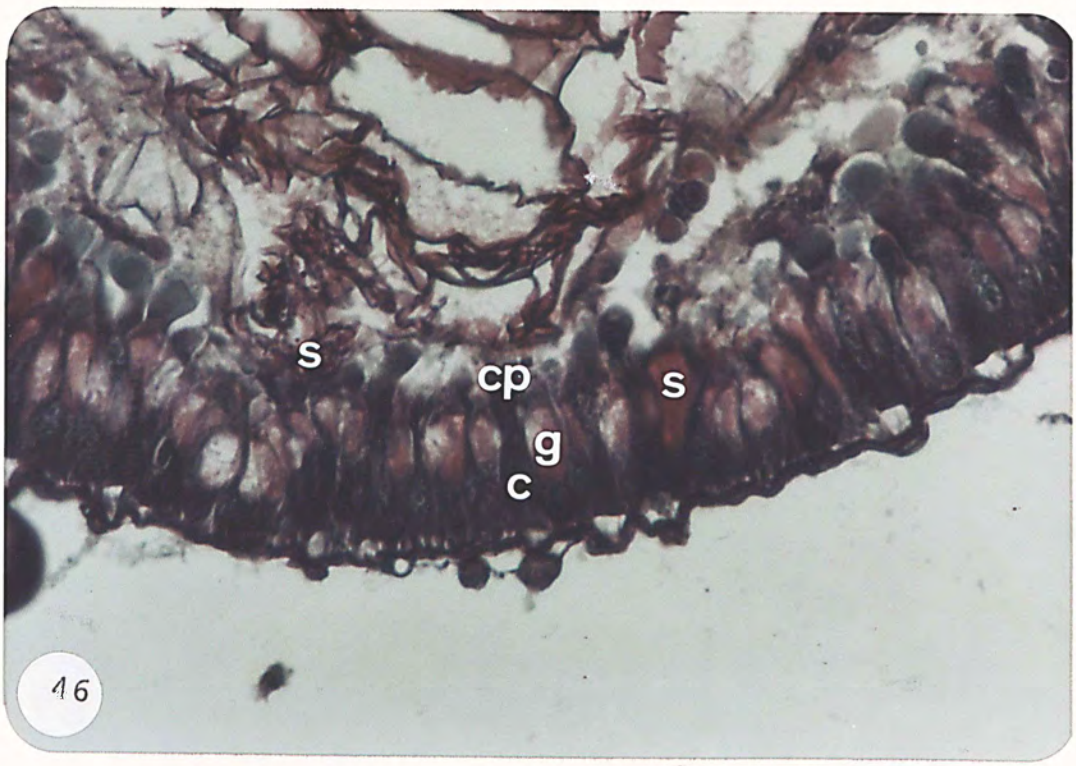
Fig. 46 Transverse section of the midgut epithelium of fifth instar larva (*Pieris canidia*), 40 minutes after *B. thuringiensis* treatment. (P.A.S. stained x 400) Note the secretory materials on the surface of columnar cells and goblet cavity which were stained purple-red (positive). The extruding vesicles, the nuclei of the columnar cells and the goblet cells were stained blue. (c, columnar cell; cp, cytoplasmic projection; g, goblet cell; s, secretory material.)

Fig. 47 Transverse section of the midgut epithelium of fifth instar larva (*Pieris canidia*), 40 minutes after *B. thuringiensis* treatment. (Alcian Blue stained x 400) The acid mucin and sulfide containing mucin were stained blue, no positive result was found on the epithelium. The secretory substances in the goblet cavities were deeply stained by safaranin (arrowed). (cp, cytoplasmic projection; g, goblet cell)


s
cp
s
g
c
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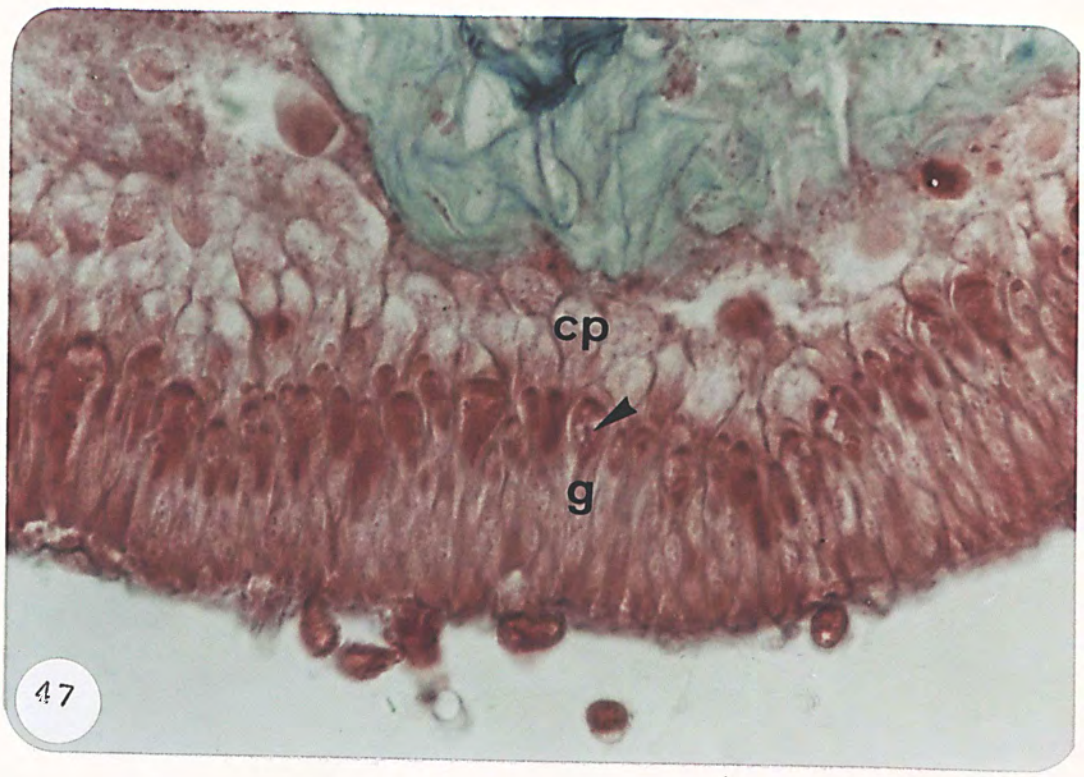
cp
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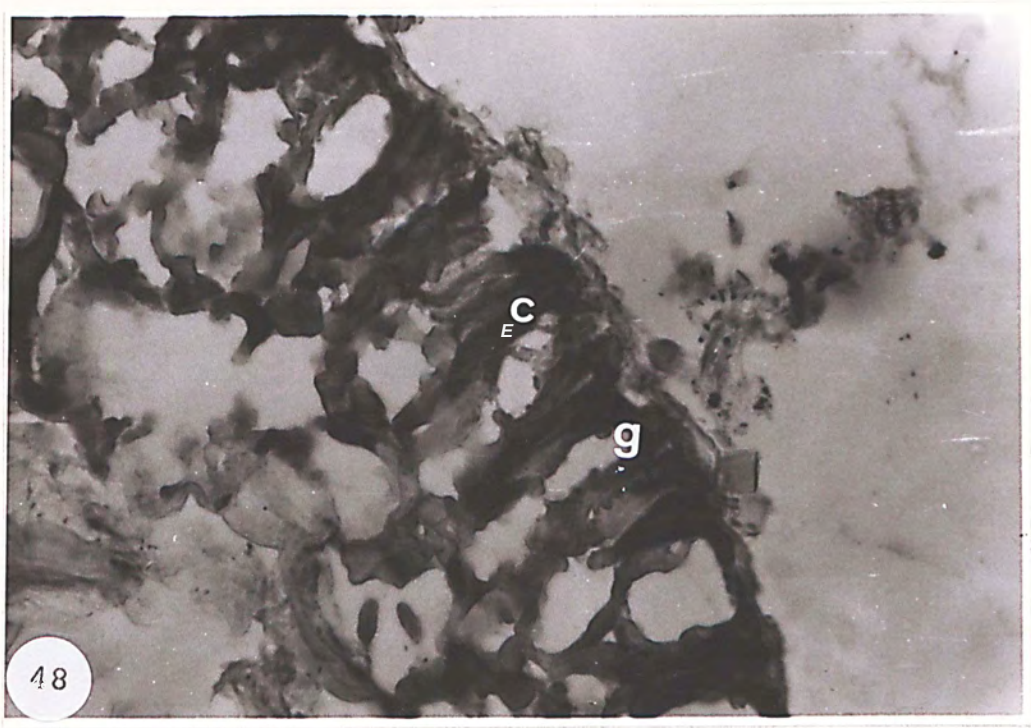
Fig. 48 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia), 40 minutes after B. thuringiensis treatment. (Sudan Black stained x 400) No lipids droplets could be detected. (c, columnar cell; g, gobiet cell)

Fig. 49 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia), 40 minutes after B. thuringiensis treatment. (Substituted Naphthol stained x 400) The activity of alkaline phosphatase was demonstrated by precipitation of a red azo dye. No positive reaction was found in the midgut epithelium. (c, columnar cell)


c
E
g
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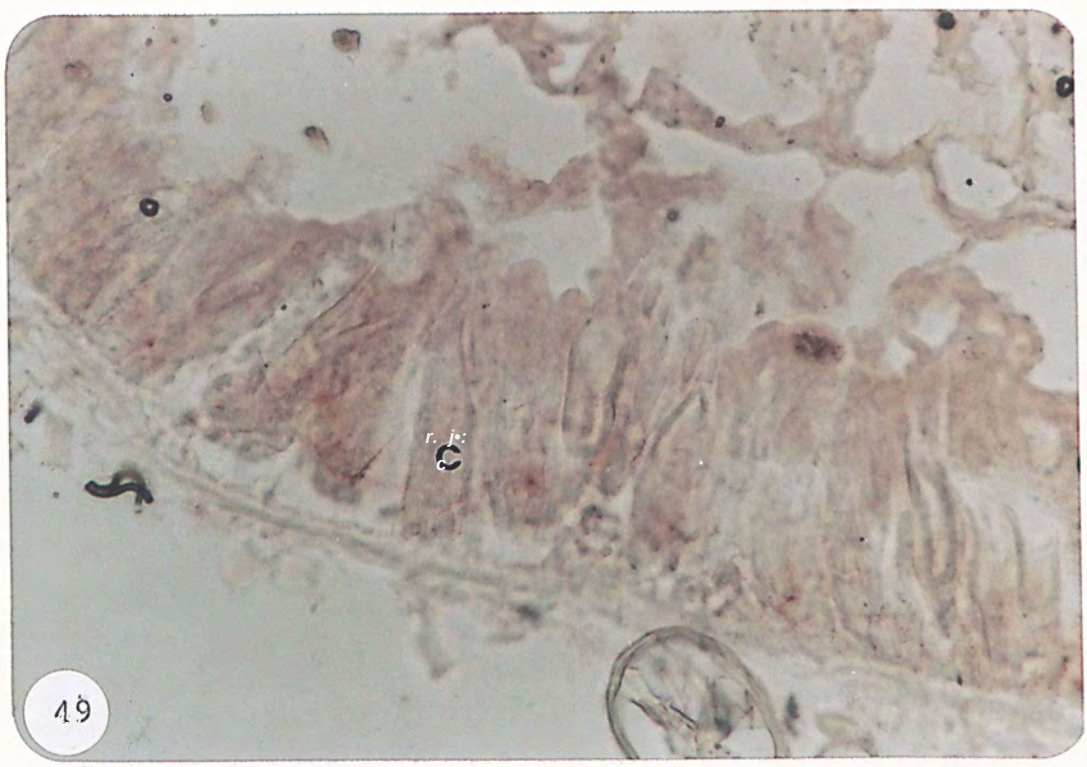
c
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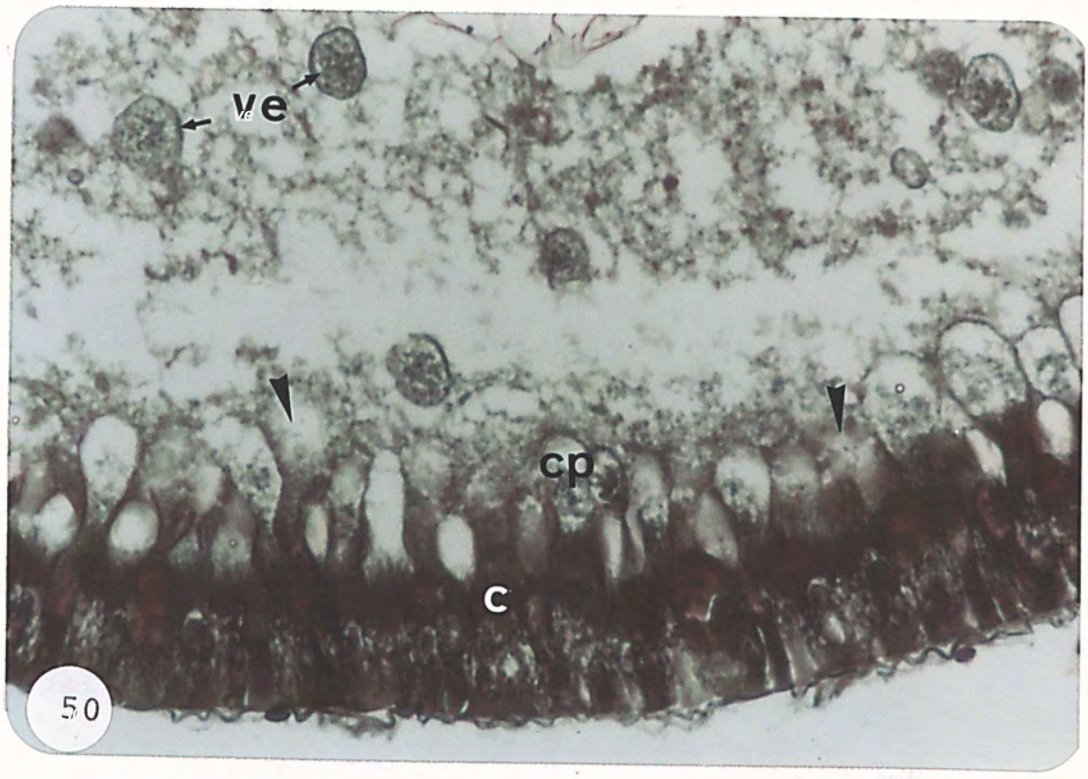
Fig. 50 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), one hour after B. thuringiensis treatment. (Mercury-Bromophenol blue stained x 400) Protein constituents were stained blue to brown. Note the extrusion of cytoplasm in the midgut epithelium (arrowed). (c, columnar cell; cp, cytoplasmic projection; ve, extruded vesicle)

Fig. 51 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), one hour after B. thuringiensis treatment. (Acid solochrome cyanine stained x 400) Basic protein was stained red. Note the high level of basic protein in the epithelium. (c, columnar cell; g, goblet cell)


ve
cp
c
50


c
g
51

Fig. 52 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), one hour after B. thuringiensis treatment. (P.A.S. stained x 400) Note the secretory materials on the surface of columnar cells and goblet cavities which were stained purple-red (positive). The extruding cytplasmic vesicles, the nuclei of the columnar cells and the goblet cells were stained blue. (c, columnar cell; cp, cytoplasmic projection; g, goblet cell; s, secretory materials; ve, extruded vesicle)

Fig. 53 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), one hour after B. thuringiensis treatment. (Alcian Blue stained x 400) The acid mucin and sulfide containing mucin were stained blue, no positive result was found on the epithelium. (c, columnar cell; cp, cytoplasmic projection; ve, extruded vesicle)


ve
cp
s
s
s
c
52

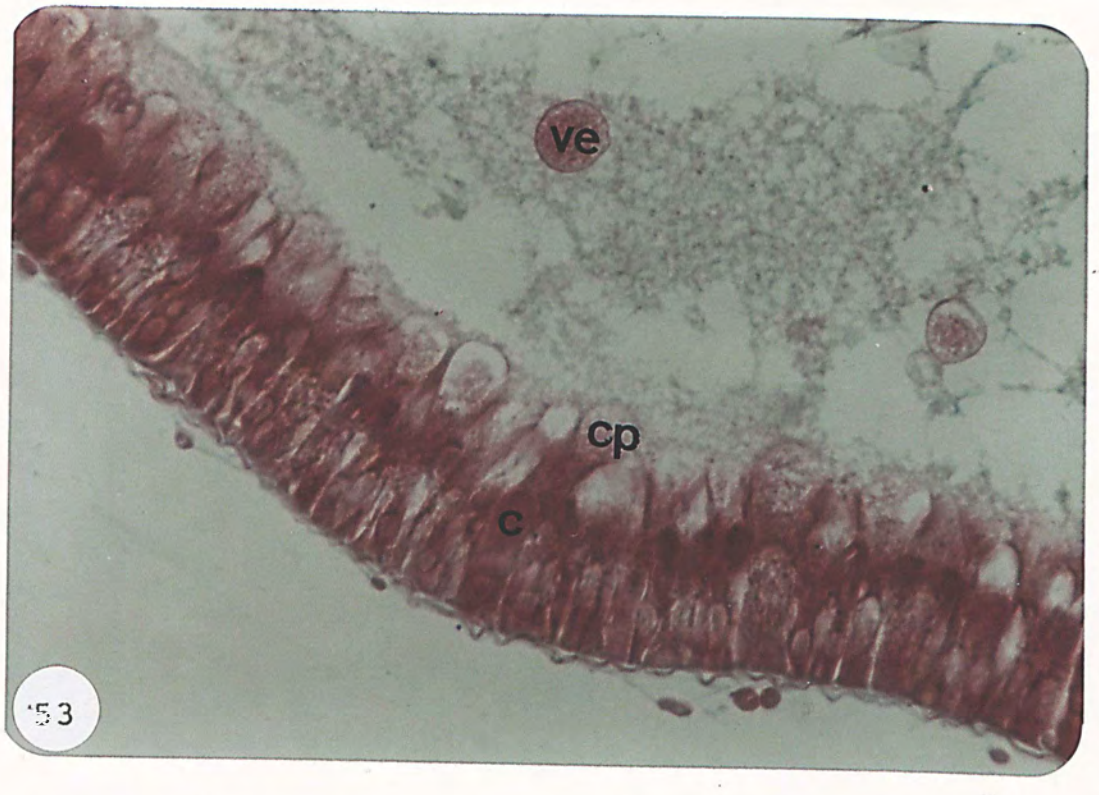
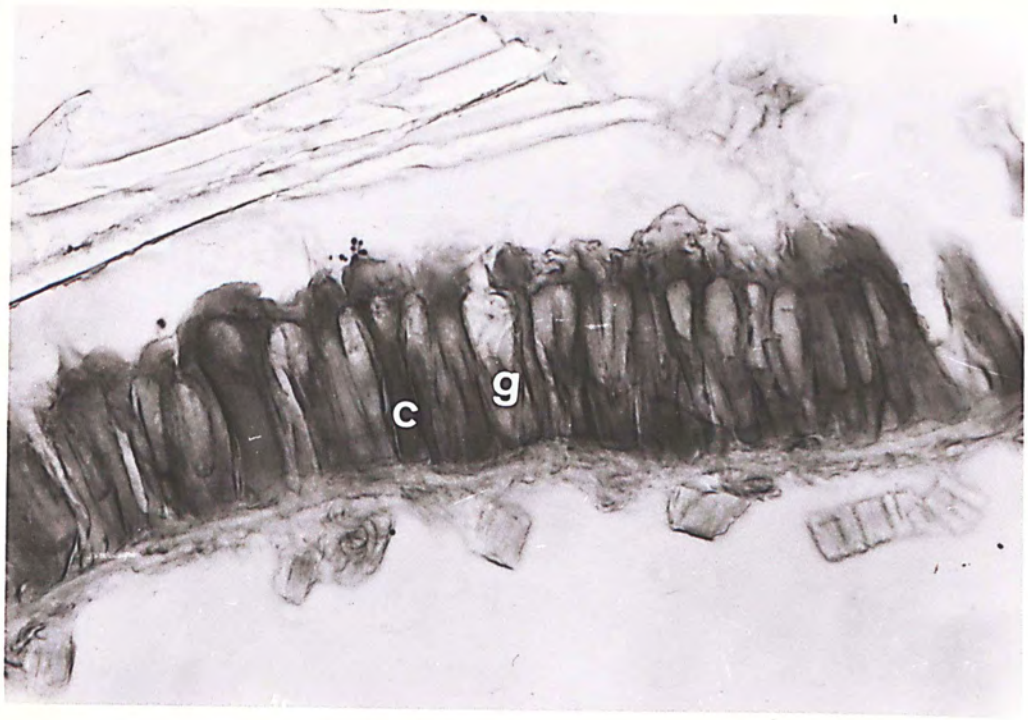

ve
cp
c
53

Fig. 54 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia), one hour after B. thuringiensis treatment. (Sudan Black stained x 400) No lipid droplets could be detected. (c, columnar cell; g, goblet cell)

Fig. 55 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia) one hour after B. thuringiensis treatment. (Substituted Naphthol stained x 400) The activity of alkaline phosphatase was demonstrated by precipitation of a red azo dye. The activity of alkaline phosphatase was detected from the centre of the columnar cell to its apex (arrowed). (c, columnar cell)



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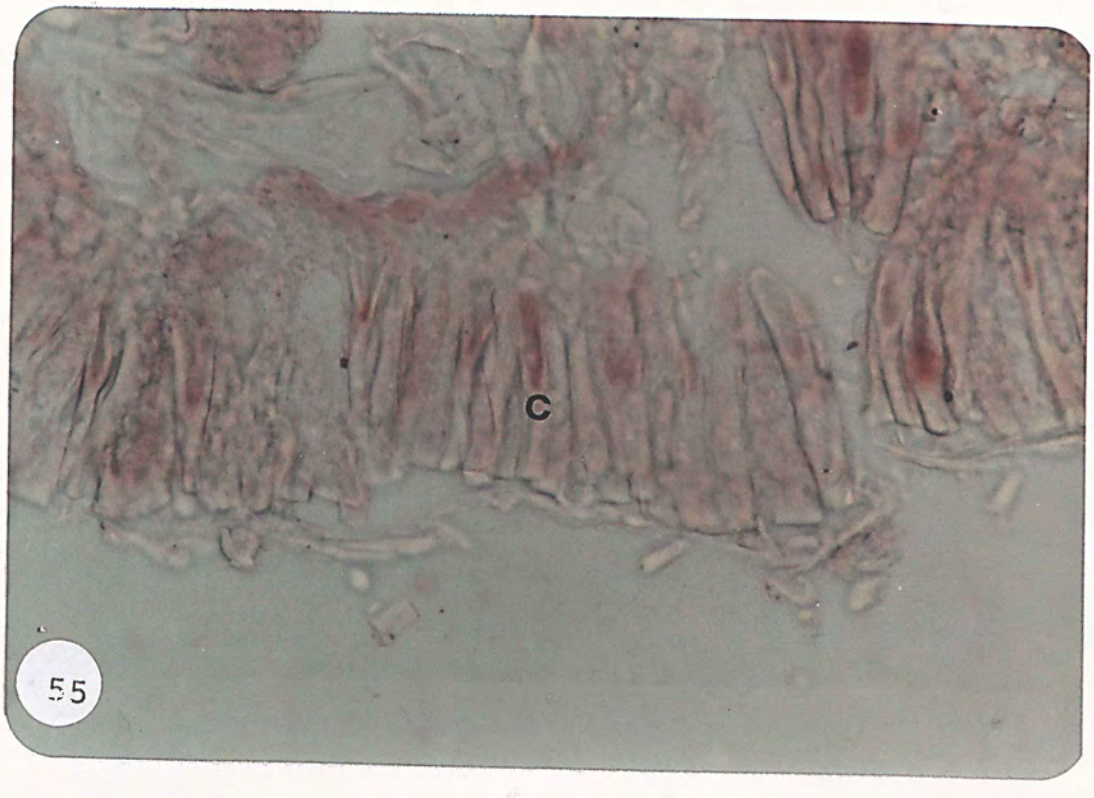

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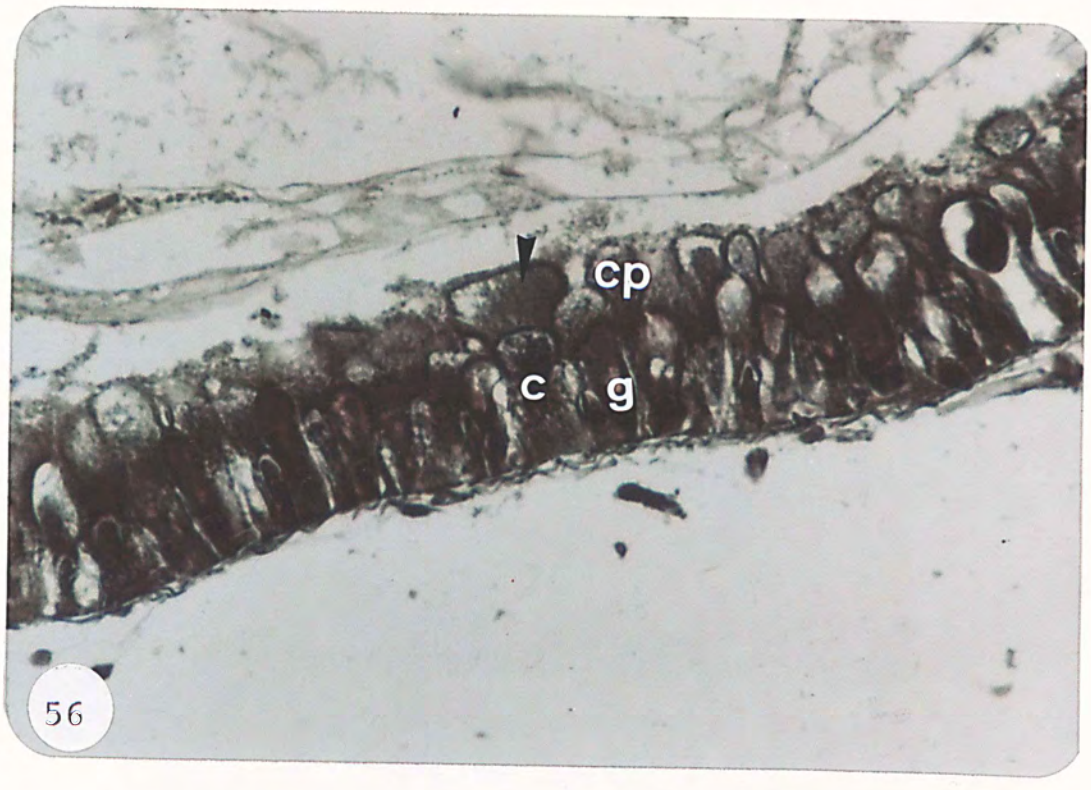
Fig. 56 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), two hours after B. thuringiensis treatment. (Mercury-Bromophenol blue stained x 400) Protein constituents were stained blue to brown. Note the extrusion of vesicles from the cytoplasm to the gut lumen. Swelling of columnar cell was observed (arrowed). (c, columnar cell; cp, cyotplasmic projection; g, goblet cell)

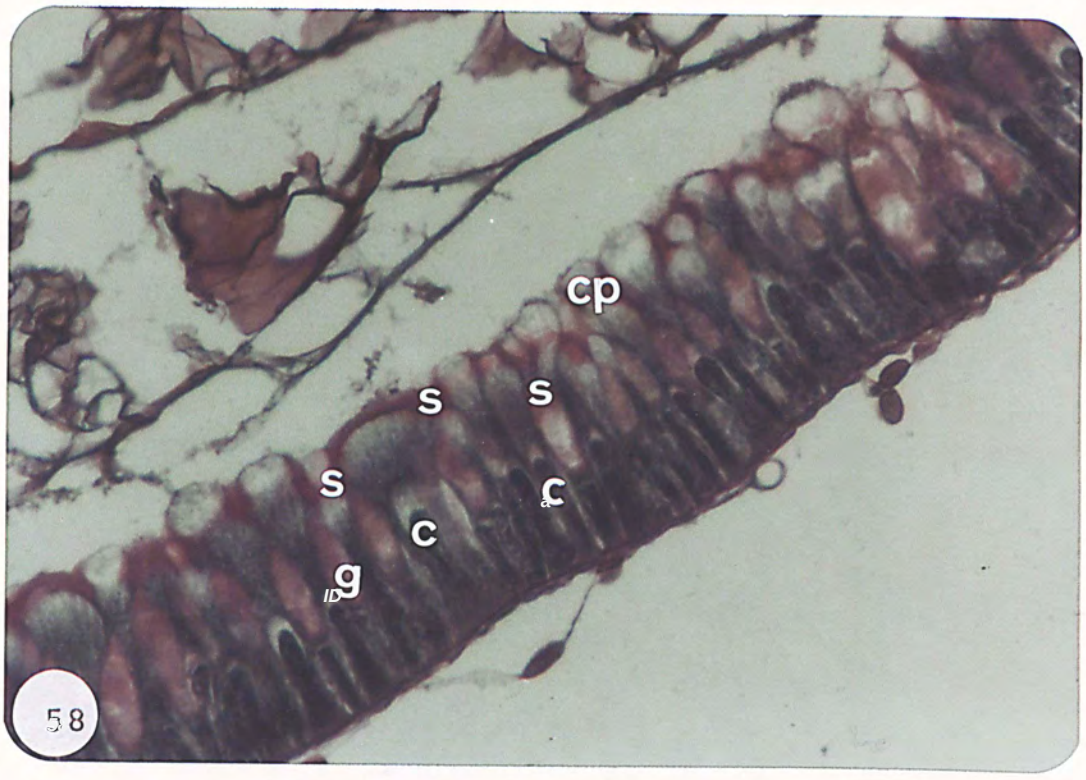
Fig. 57 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), two hours after B. thuringiensis treatment. (Acid solochrome cyanine stained x 400) Basic protein was stained red. Note the high level of basic protein in the epithelium. (c, columnar cell; cp, cytoplasmic projection; g, goblet cell)


cp
c
g
56


cp
c
g
57

Fig. 58 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), two hours after B. thuringiensis treatment. (P.A.S. stained x 400) Note the secretous materials on the surface of columnar cells, and goblet cavities which were stained purple-red (positive). The nuclei of the columnar cells and the goblet cells were stained blue. (c, columnar cell; cp, cytoplasmic projection; g, goblet cell; s, secretory materials)

Fig. 59 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia), two hours after B. thuringiensis treatment. (Alcian Blue stained x 400) The acid mucin and sulfide containing mucin were stained blue, no positive result was found on the epithelium. (c, columnar cell; g, goblet cell)


cp
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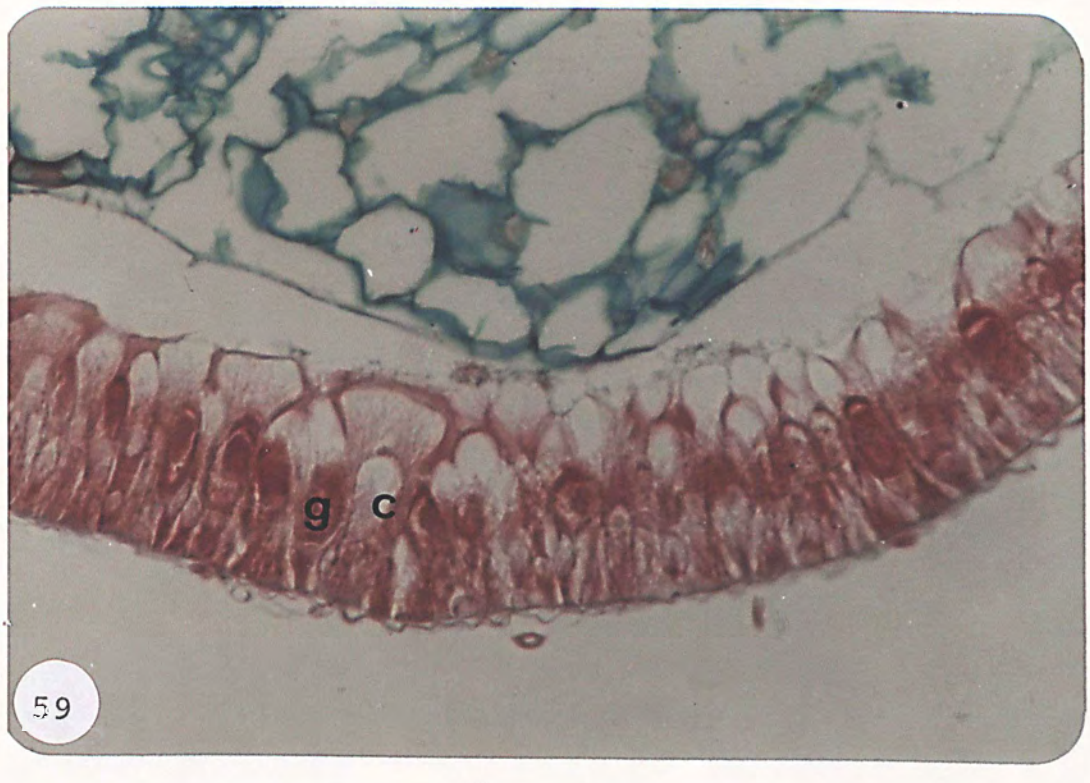
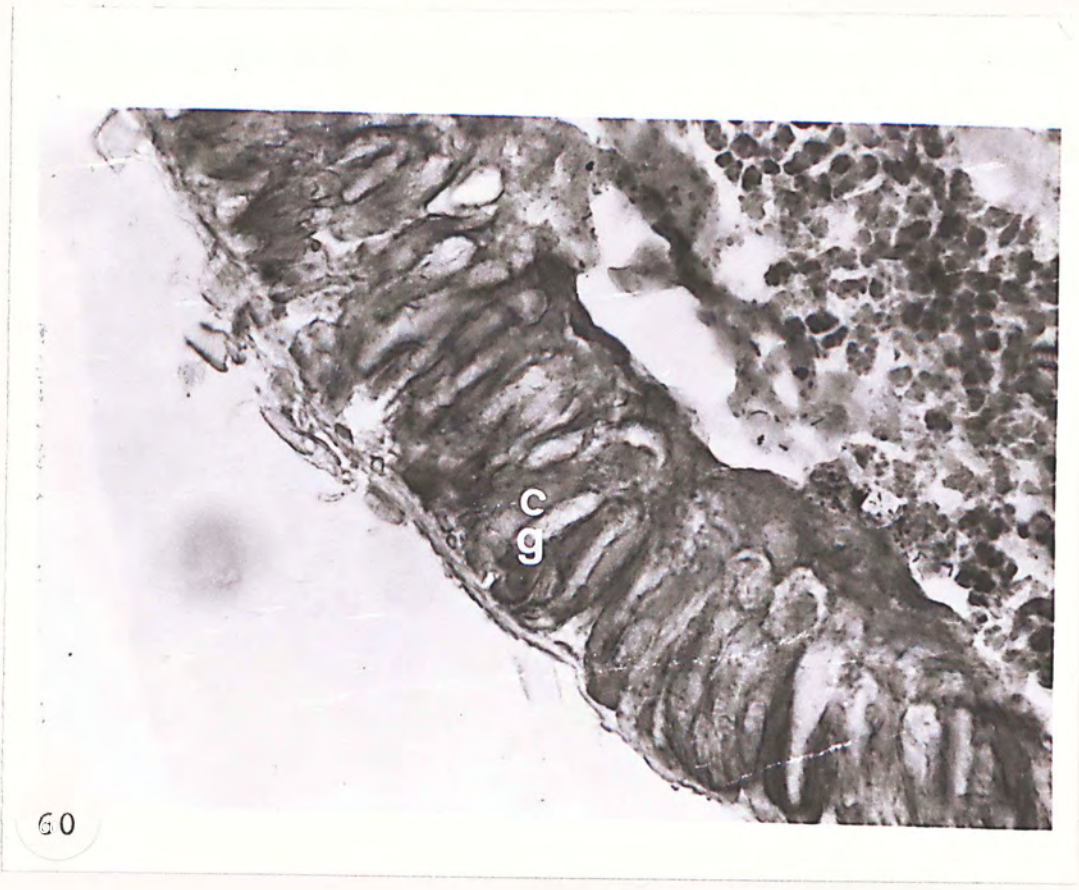

g
c
59

Fig. 60 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia), two hours after B. thuringiensis treatment. (Sudan Black stained x 400) No lipids droplets could be detected. (c, columnar cell; g, goblet cell)

Fig. 61 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia), two hours after B. thuringiensis treatment. (Substituted Naphthol stained x 400) The activity of alkaline phosphatase was demonstrated by precipitation of a red azo dye. The activity of alkaline phosphatase was found at the apices of columnar cells (arrowed). (c, columnar cell)



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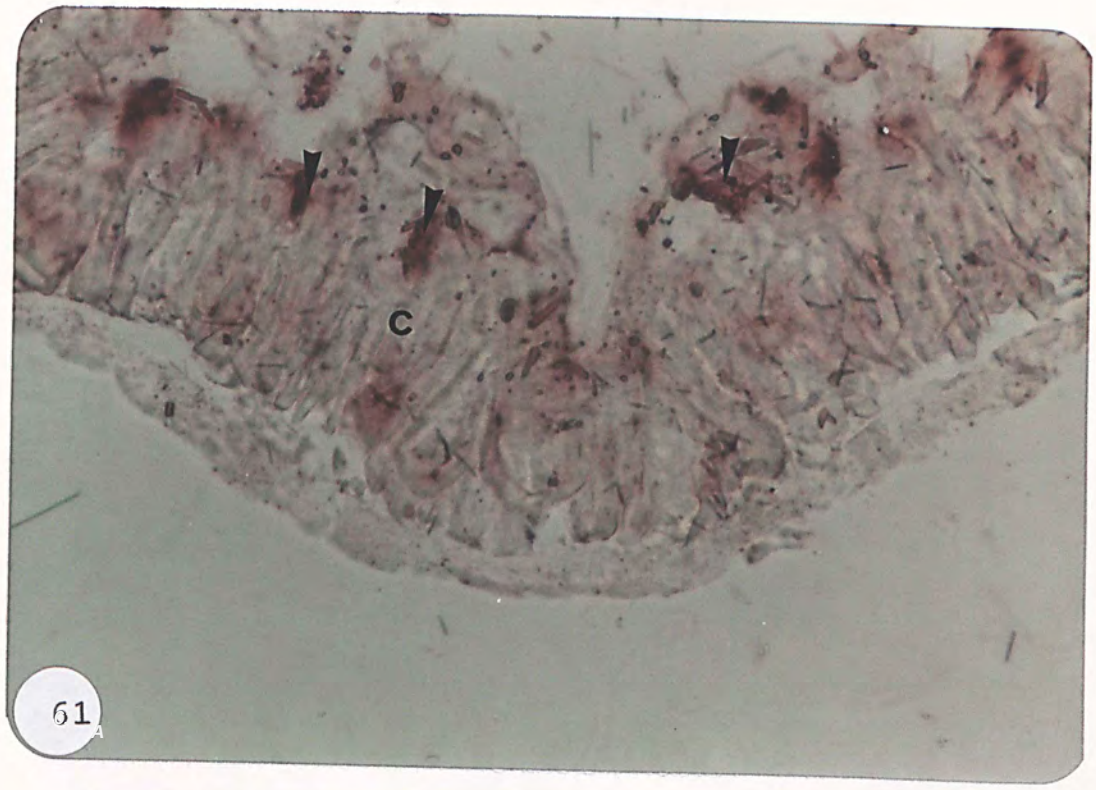
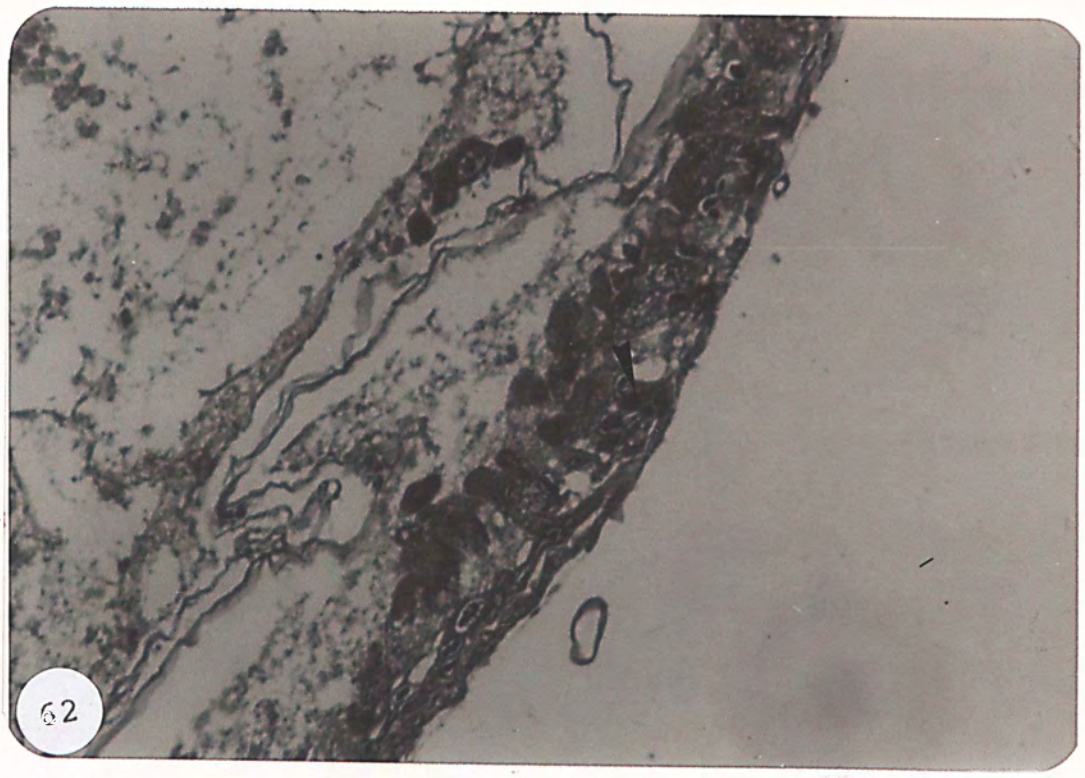


61

Fig. 62 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) three hours after B. thuringiensis treatment. (Mercury-Bromophenol blue stained x 200) Protein constituents were stained blue to brown. note that the columnar cells lysed, the goblet cells swelled and they sloughed off from the midgut epithelium (arrowed).

Fig. 63 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) three hours after B. thuringiensis treatment. (Acid solochrome cyanine stained x 200) Basic protein was stained red. Note the high level of basic protein in the epithelium. (c, columnar cell; g, goblet cell)



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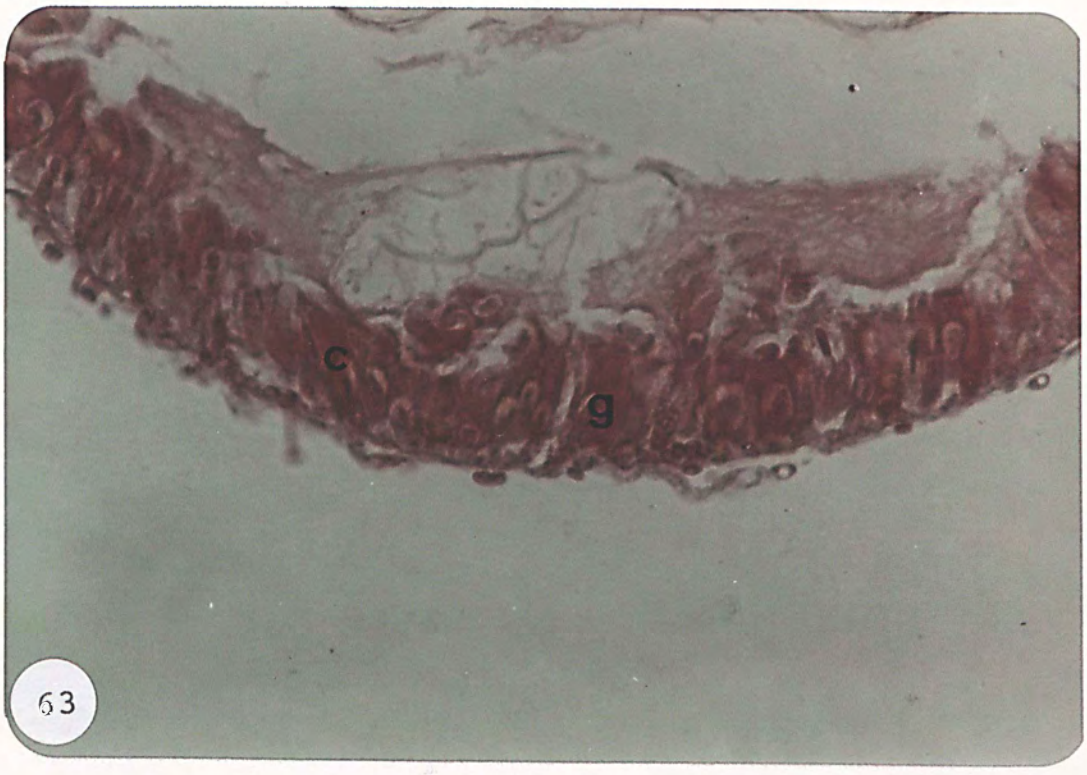

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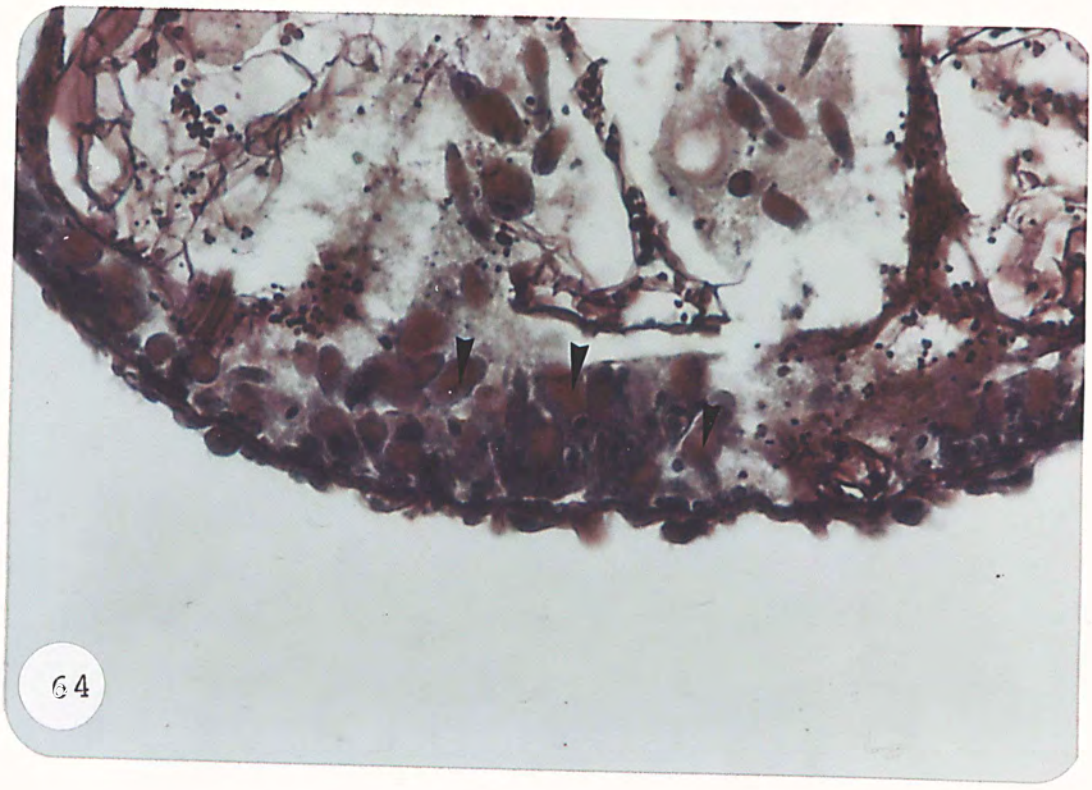
Fig. 64 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) three hours after B. thuringiensis treatment. (P.A.S. stained x 200) Note the disintegration of the mucous layer, the swollen goblet cells (arrowed) were stained purple-red (positive), and they sloughed off from the epithelium.

Fig. 65 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) three hours after B. thuringiensis treatment. (Alcian Blue stained x 200) No positive result was found on the epithelium. (g, goblet cell; tr, tracheole)



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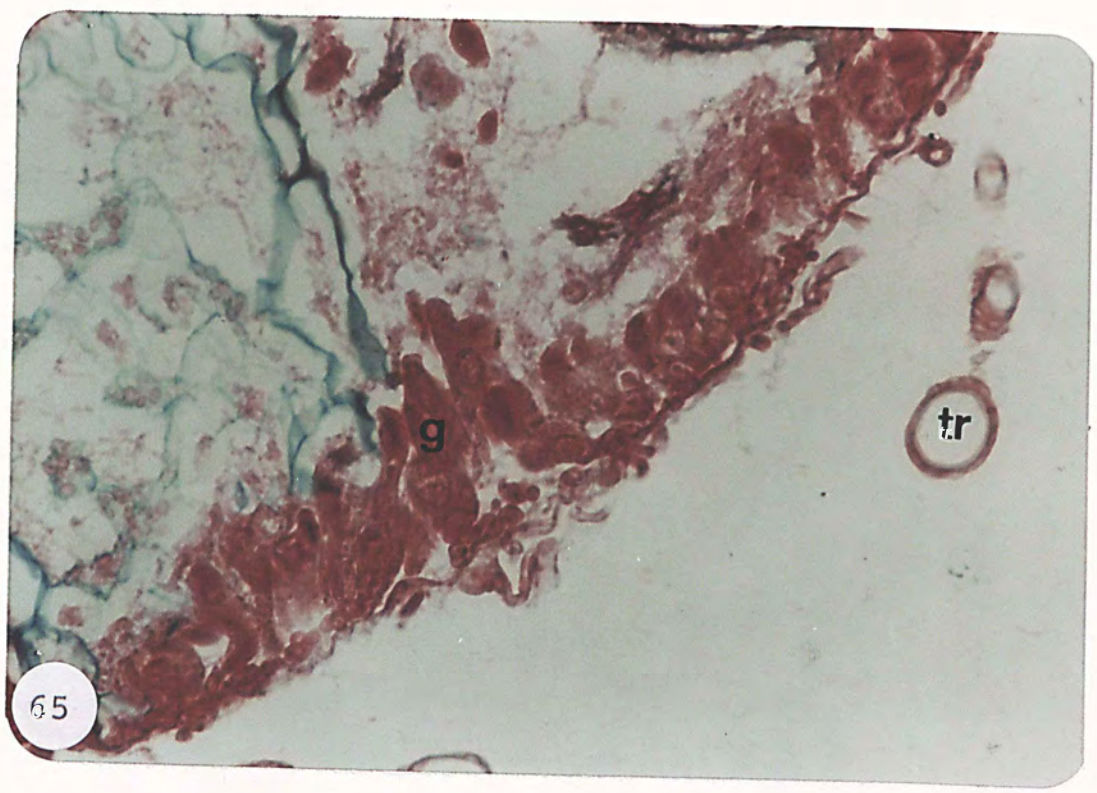

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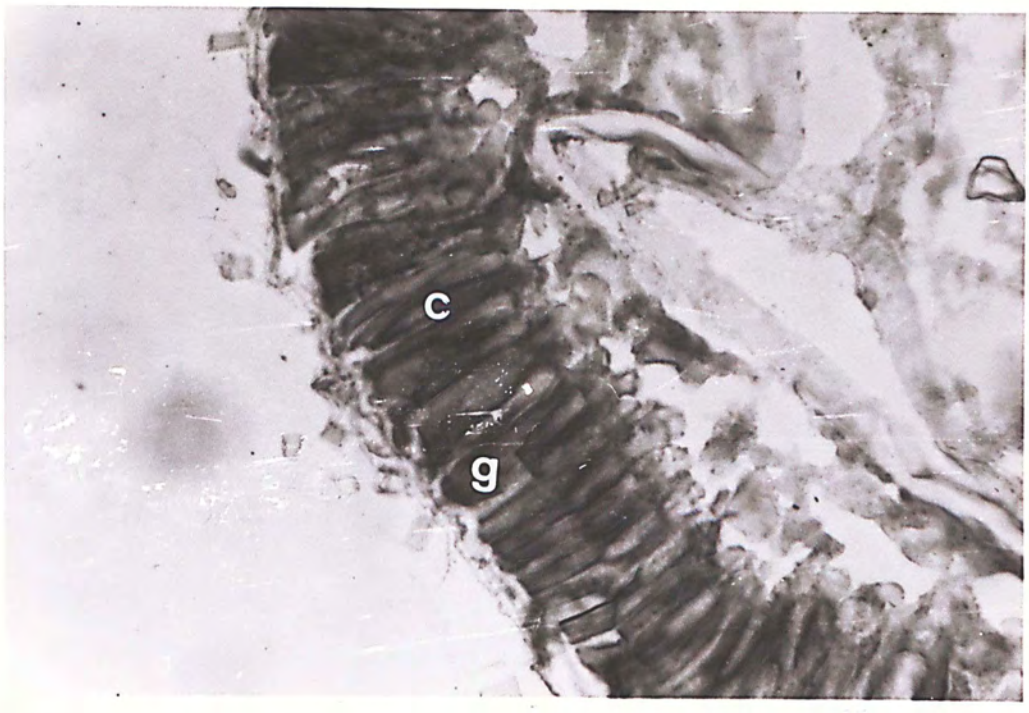
Fig. 66 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia) three hours after B. thuringiensis treatment. (Sudan Black stained x 400) No lipid droplets could be detected. (c, columnar cell; g, goblet cell)

Fig. 67 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia) three hours after B. thuringiensis treatment. (Substituted Naphthol stained x 400) The activity of alkaline phosphatase was demonstrated by precipitation of a red azo dye. The activity of alkaline phosphatase was found to be increased at the apices of columnar cells (arrowed). (c, columnar cell; g, goblet cell)



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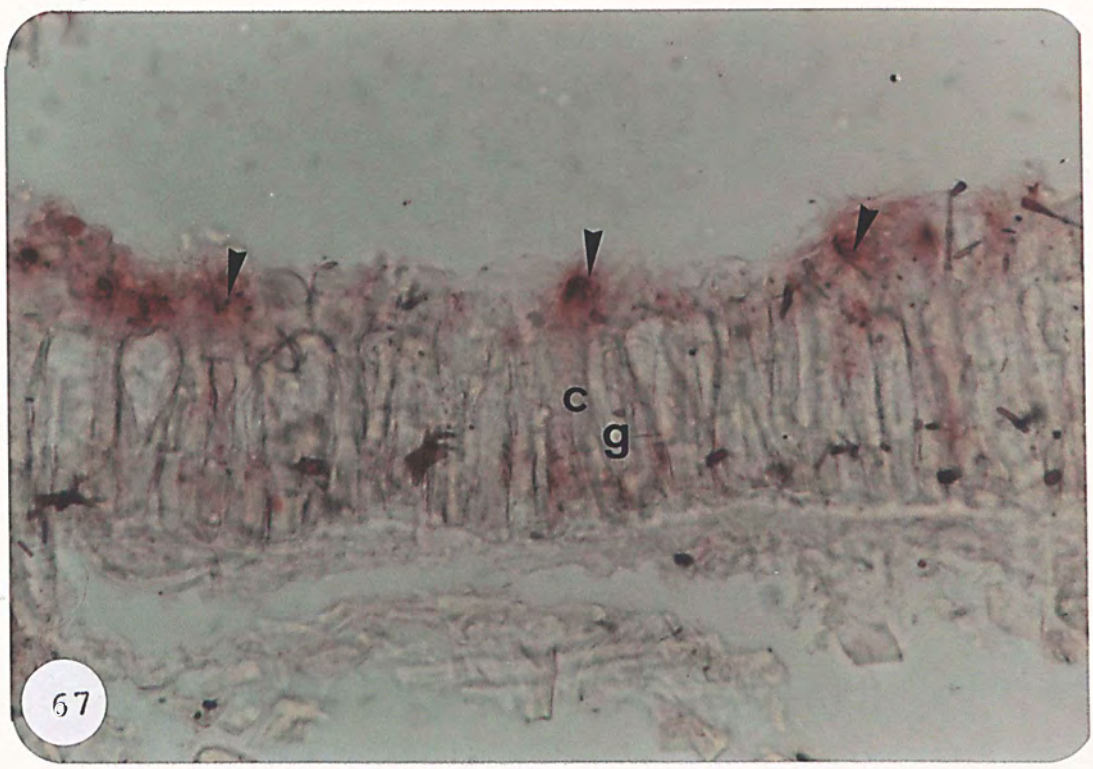

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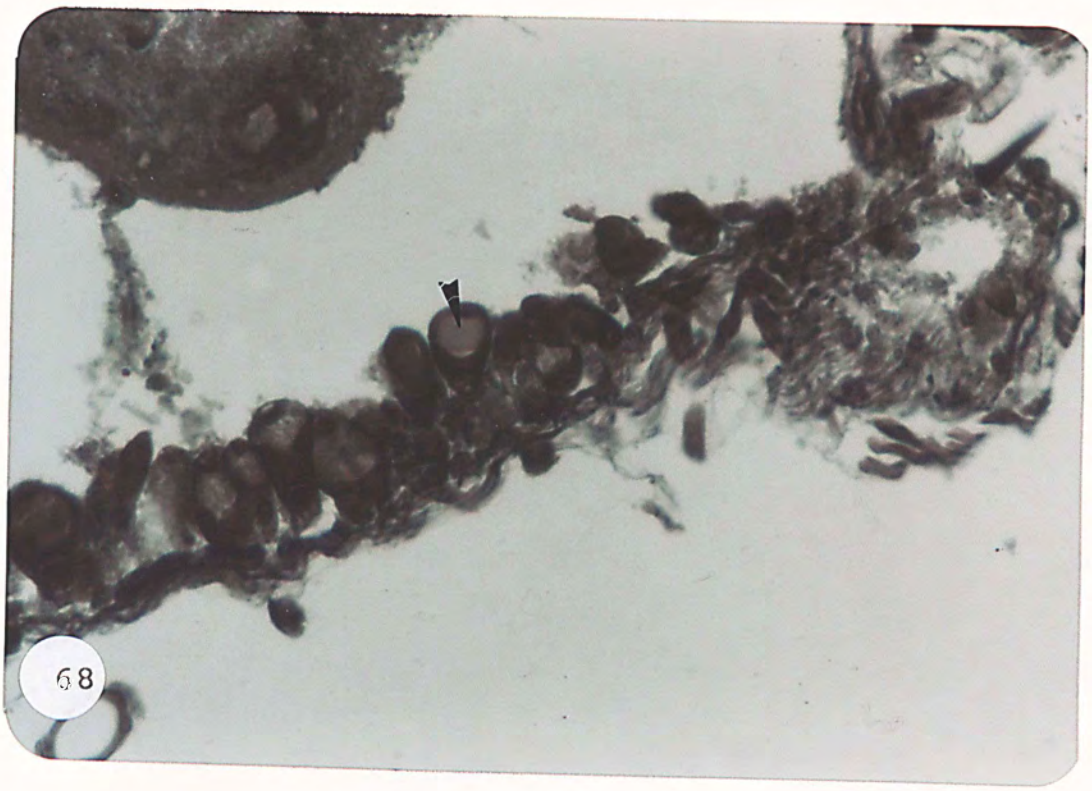
Fig. 68 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) four hours after B. thuringiensis treatment. (Mercury-Bromophenol blue stained x 400) Protein constituents were stained blue to brown. Note the swollen goblet cells (arrowed) sloughed off from the midgut epithelium.

Fig. 69 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) four hours after B. thuringiensis treatment. (Acid solochrome cyanine stained x 400) Basic protein was stained red. Note the high level of basic protein in the epithelium. (g, goblet cell)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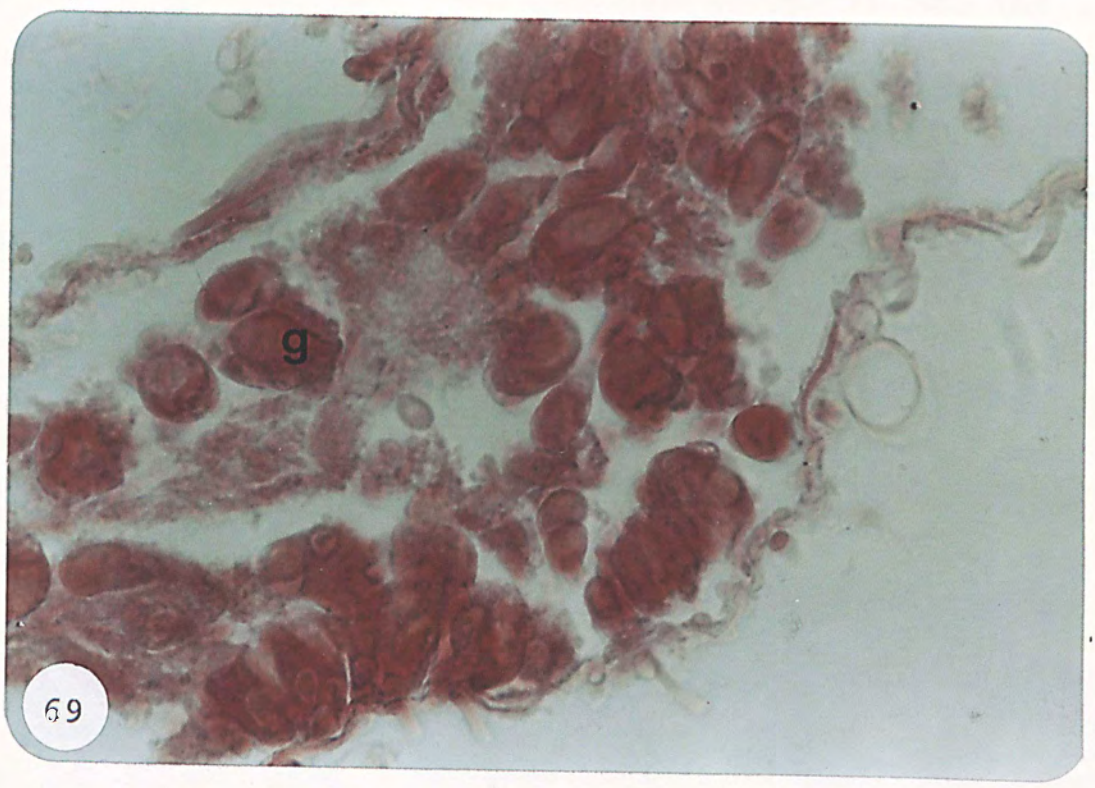
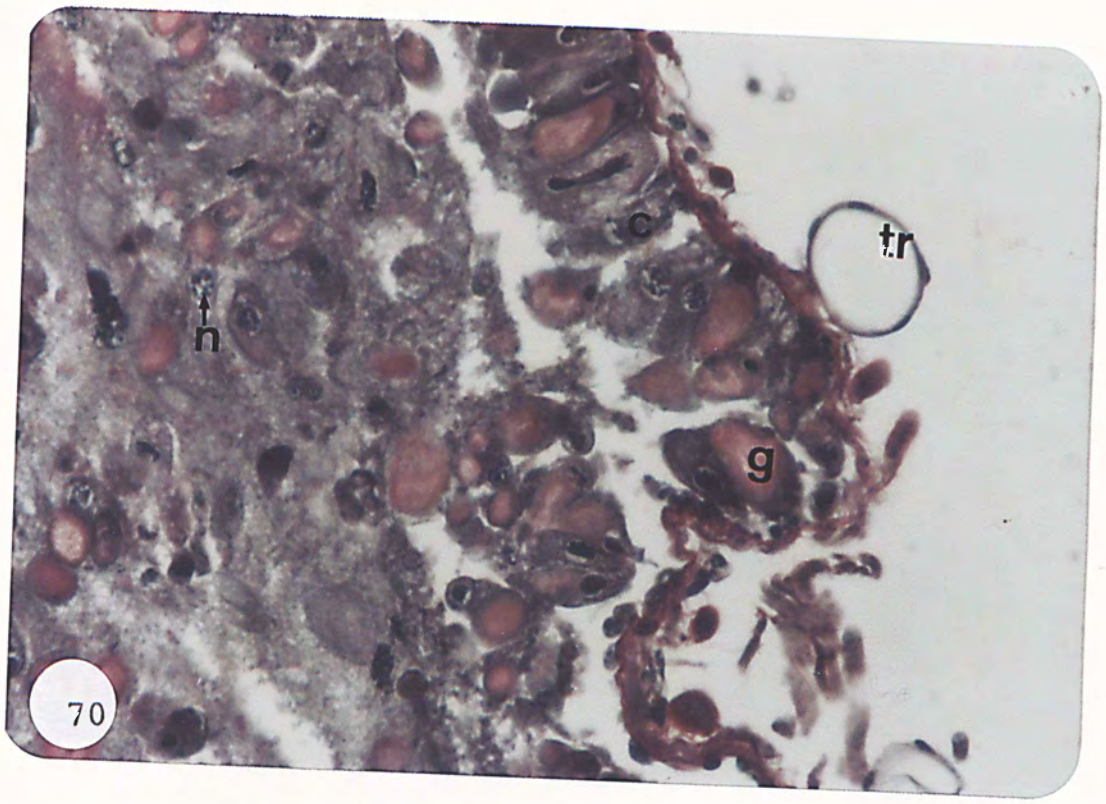

g
69

Fig. 70 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) four hours after B. thuringiensis treatment. (P.A.S. stained x 400) Note the disintegration of the midgut epithelium, the swollen goblet cells were stained purple-red (positive), they sloughed off from the epithelium. (c, columnar cell; g, goblet cell; n, nucleus; tr, tracheole)

Fig. 71 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) four hours after B. thuringiensis treatment. (Alcian Blue stained x 400) No positive result was found on the epithelium. (g, goblet cell)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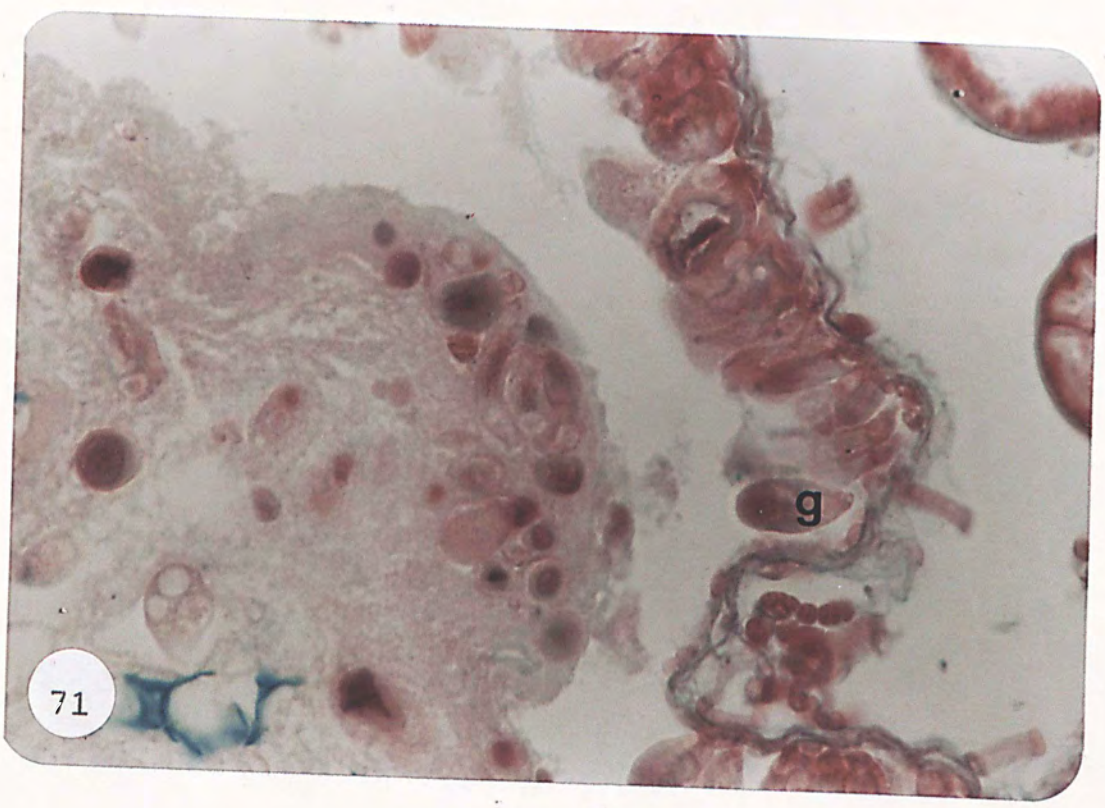
g
71

Fig. 72 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia) four hours after B. thuringiensis treatment. (Sudan Black stained x 400) No lipid droplets could be detected. (m, muscle sheath)

Fig. 73 Cryostat section of the midgut epithelium of fifth instar larva (Pieris canidia) four hours after B. thuringiensis treatment. (Substituted Naphthol stained x 400) The activity of alkaline phosphatase was demonstrated by precipitation of a red azo dye. The activity of alkaline phosphatase was lost as the epithelial cells lysed. (m, muscle sheath)


m
72

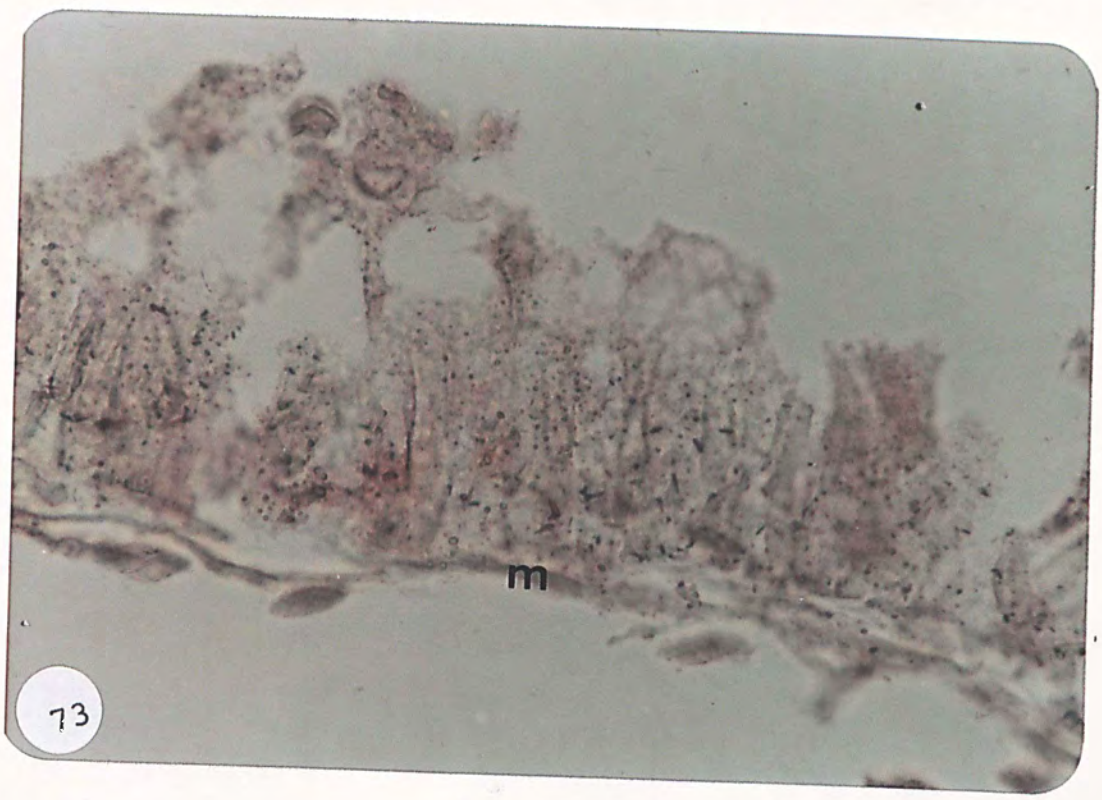
m
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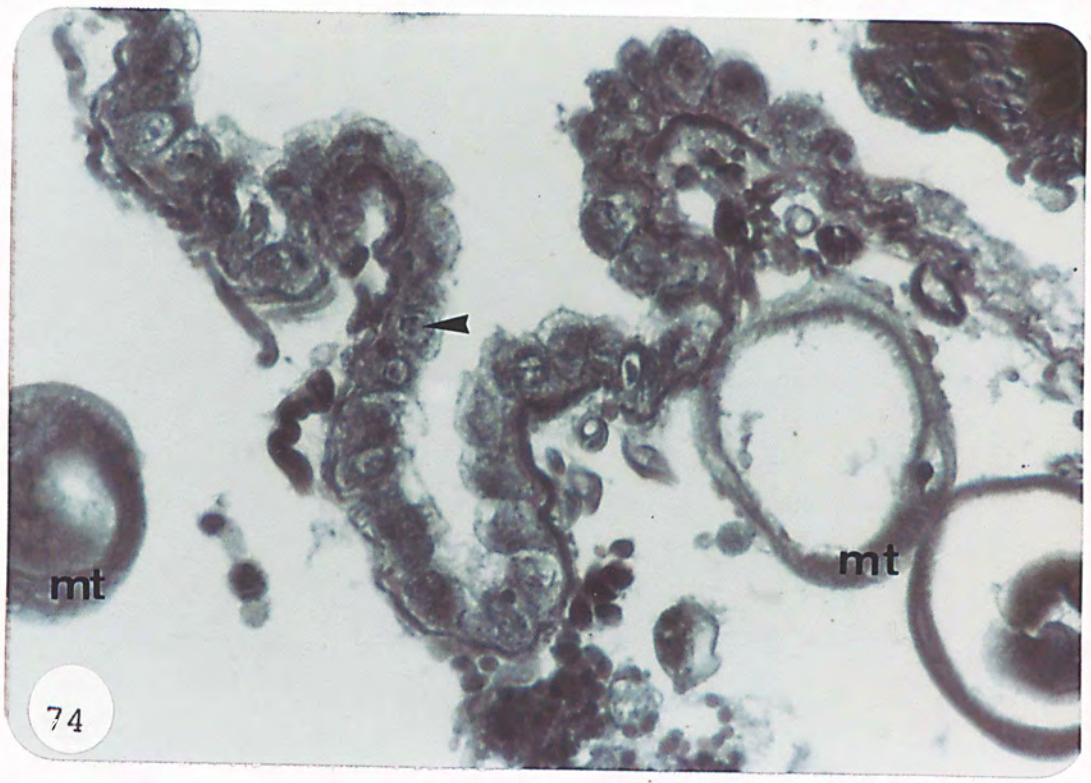
Fig. 74 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) five hours after B. thuringiensis treatment. (Mercury-Bromophenol blue stained x 400) Protein constituents were stained blue. All the epithelial cells lysed. A layer of cubodial cells (arrowed) was found on the epitheilum. (mt, malpighian tubule)

Fig. 75 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) five hours after B. thuringiensis treatment. (Acid solochrome cyanine stained x 400) Basic protein was stained red. Note the particularly low level of basic protein in the epithelium. (n, nucleus)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mt
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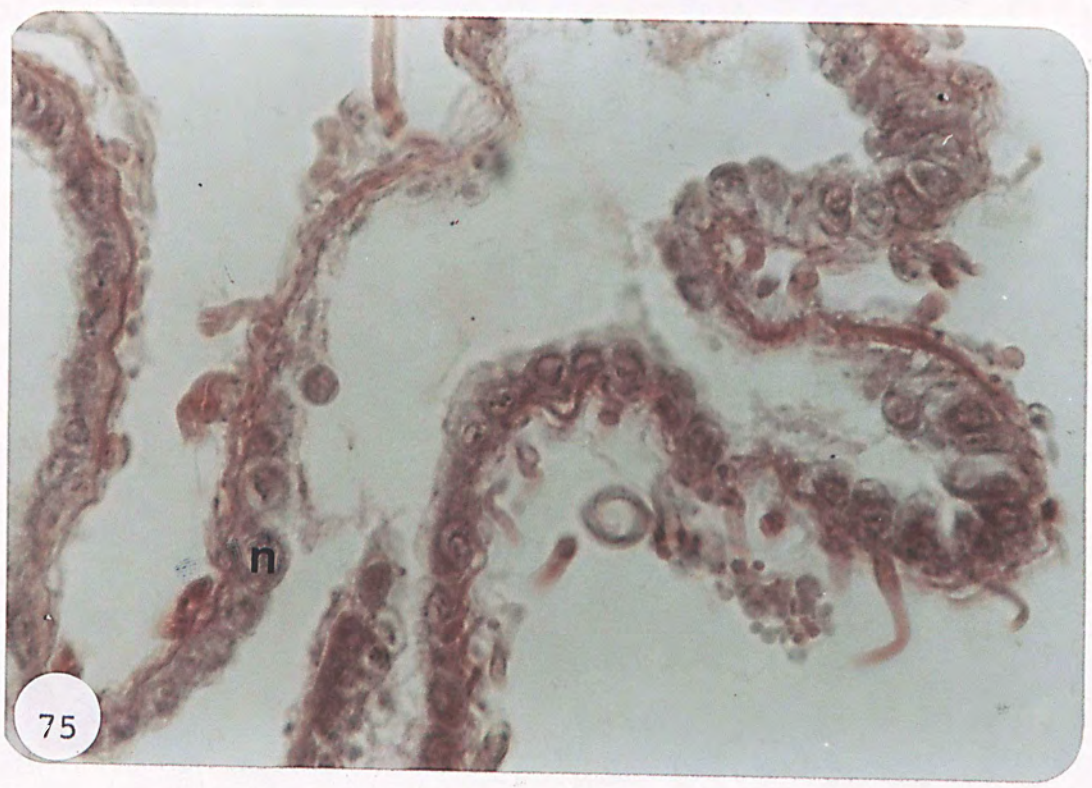
n
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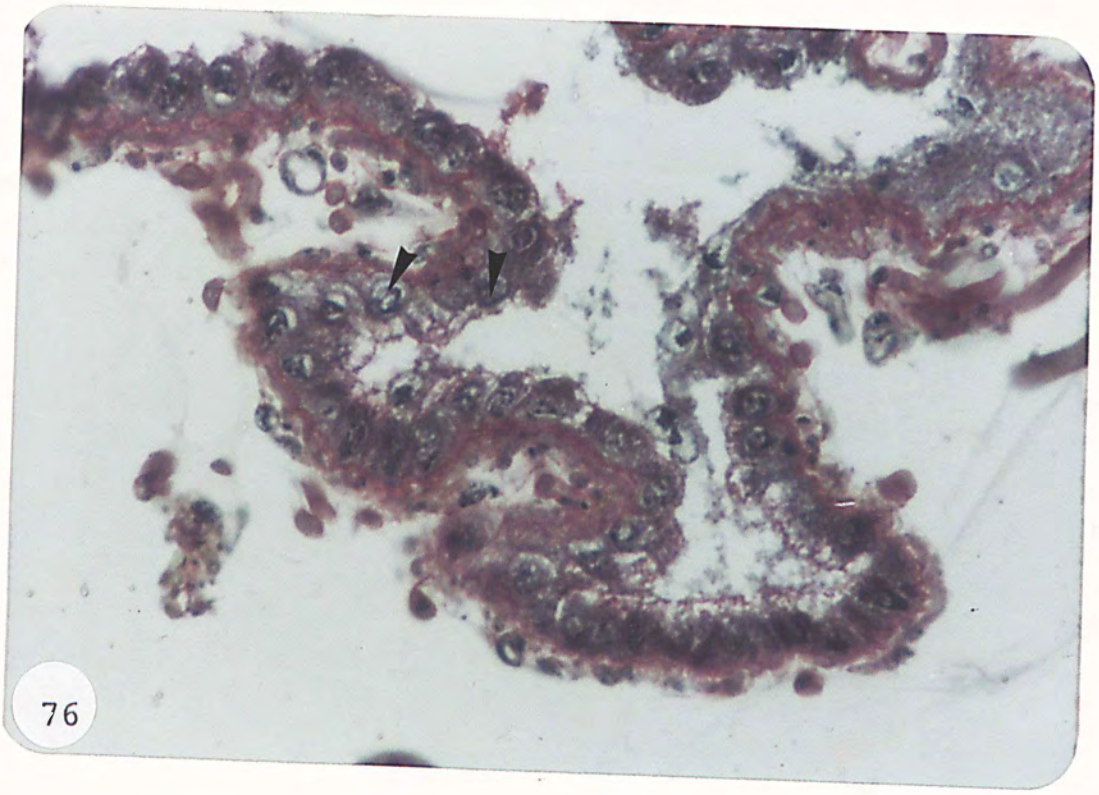
Fig. 76 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) five hours after B. thuringiensis treatment. (P.A.S. stained x 400) Note the disintegration of the midgut epithelium, the cuboidal cells were having large nuclei (arrowed) and little cytoplasm.

Fig. 77 Transverse section of the midgut epithelium of fifth instar larva (Pieris canidia) five hours after B. thuringiensis treatment. (Alcian Blue stained x 400) No positive result was found on the epithelium.



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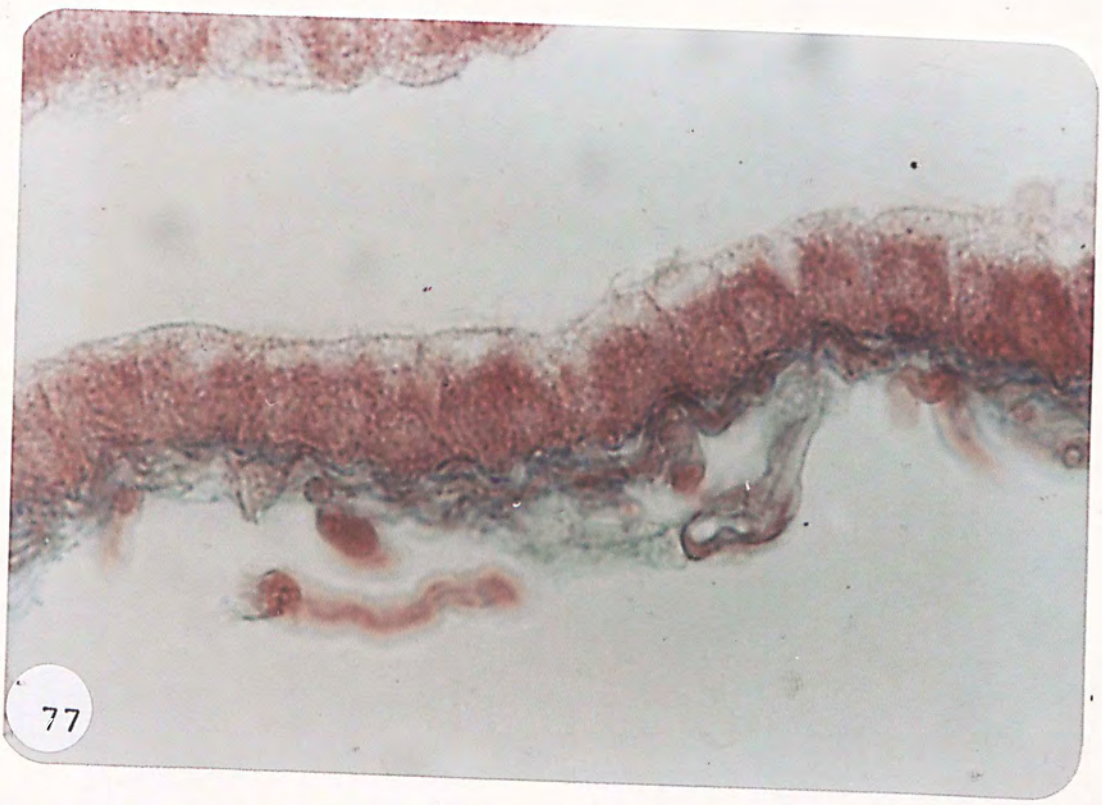

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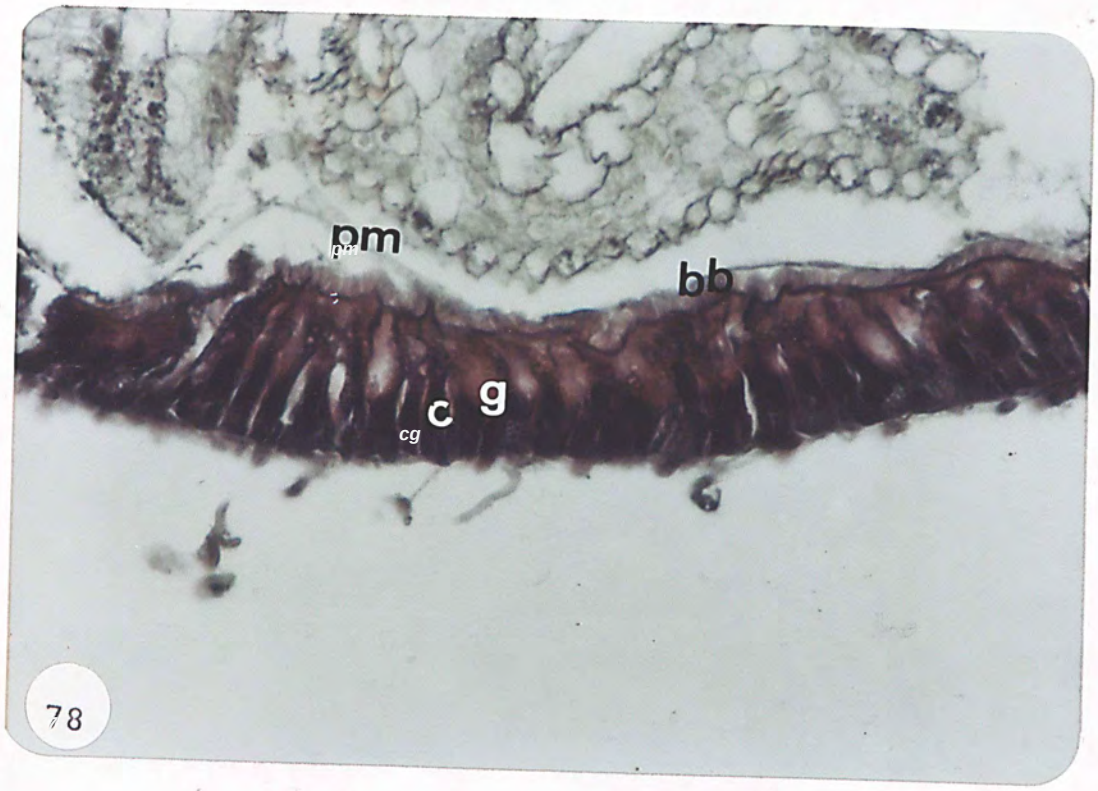
Fig. 78 Transverse section of the midgut epithelium of the fourth instar larva (Parnara guttata) without B. thuringiensis treatment. (H & E stained x 400) Note the centrally located nuclei in the columnar cells, the urn-shaped cavities within the goblet cells, and the brush border on the surface of the epithelium. The peritrophic membrane was found lining the brush border. (bb, brush border; c, columnar cell; g, goblet cell; pm, peritrophic membrane)

Fig. 79 Transverse section of the midgut epithelium of fourth instar larva (Parnara guttata) without B. thuringiensis treatment. (P.A.S. stained x 400) Note the P.A.S. positive mucous lining the brush border. (bb, brush border; n, nucleus)



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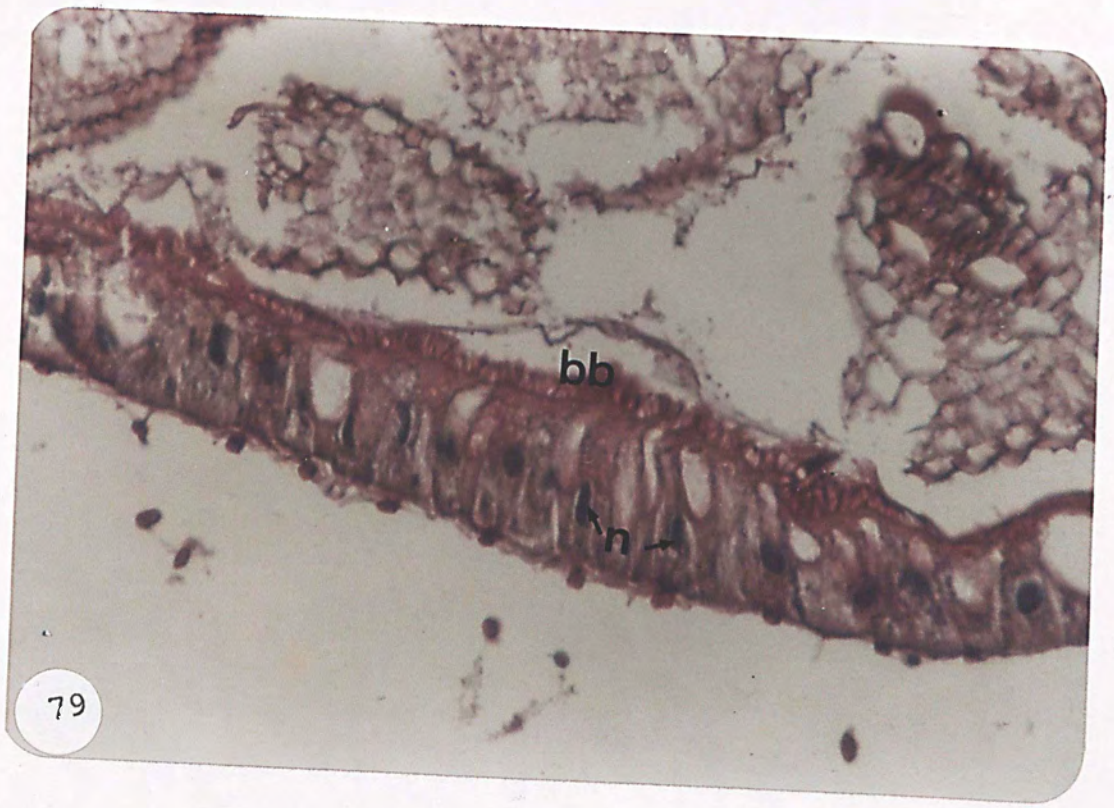

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Fig. 80 Transverse section of the midgut epithelium of the fourth instar larva (Parnara guttata) without B. thuringiensis treatment. (Mercury-Bromophenol blue stained x 400) Note the closely packed brush border (arrowed). (c, columnar cell; g, goblet cell)

Fig. 81 Transverse section of the midgut epithelium of fifth instar larva (Parnara guttata) without B. thuringiensis treatment. (Acid solochrome cyanine stained x 200) Note the low level of basic protein on the epithelium; the nuclei were stained blue (arrowed). (c, columnar cell; g, goblet cell)

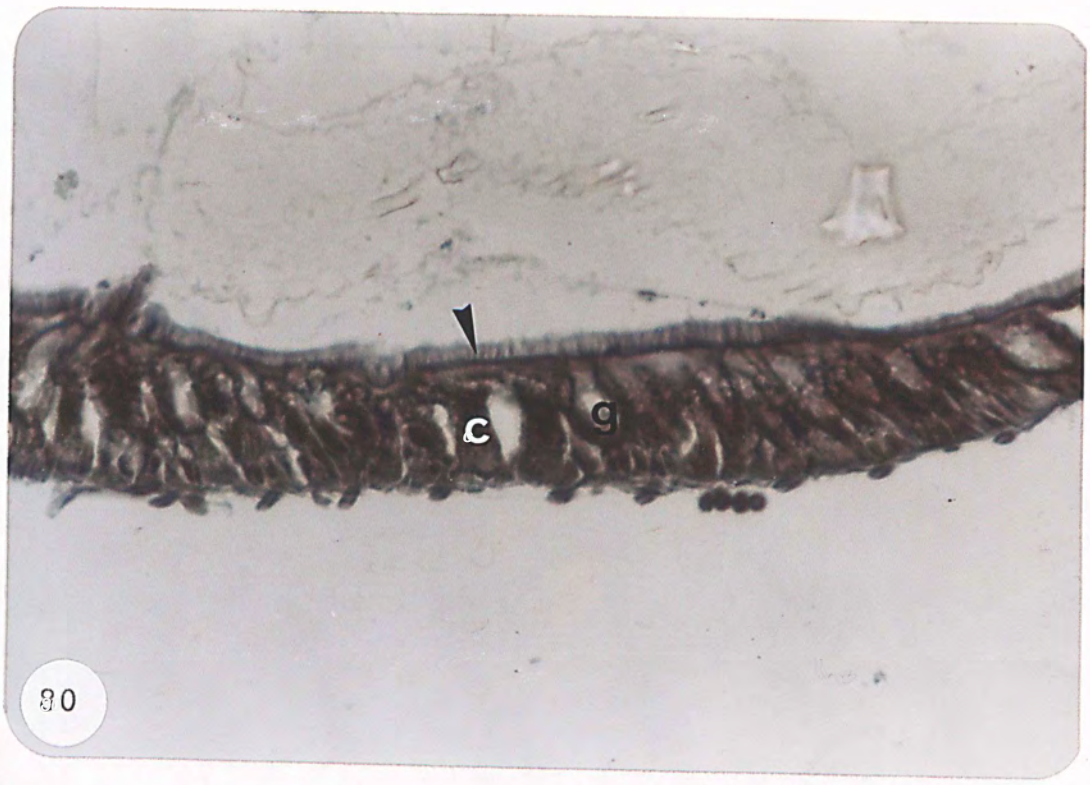
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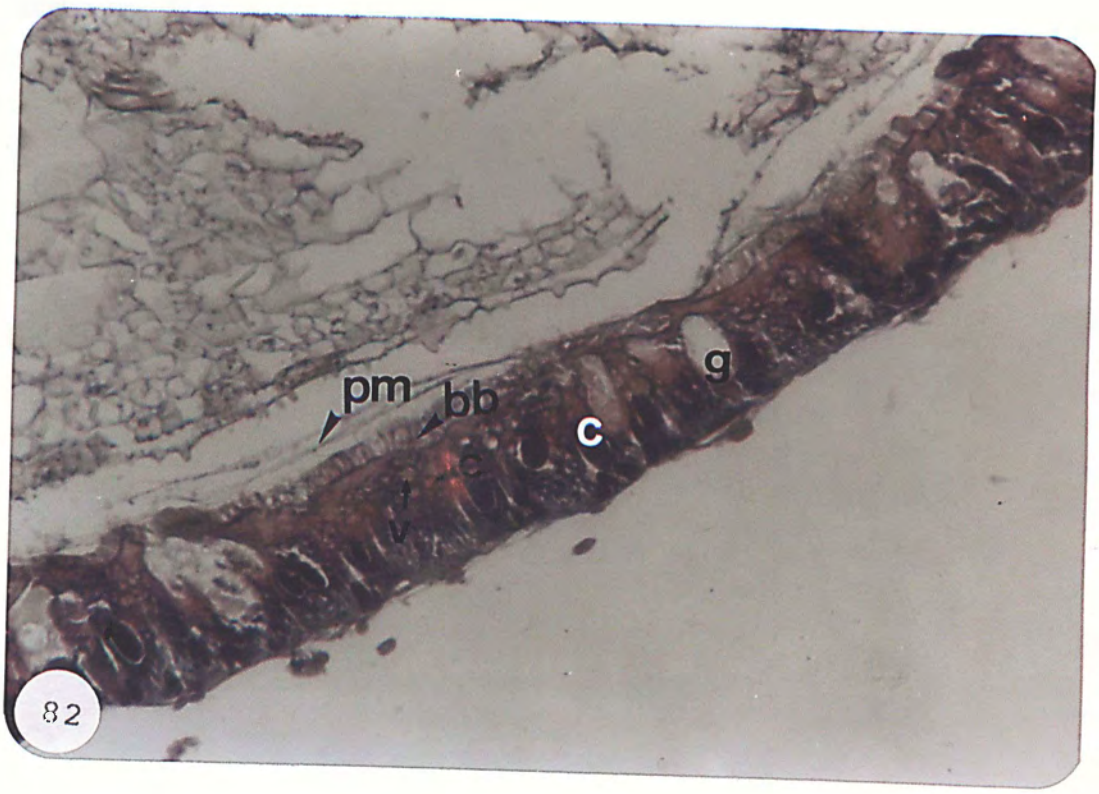
Fig. 82 Transverse section of the midgut epithelium of the fourth instar larva (Parnara guttata) one hour after B. thuringiensis treatment. (H & E stained x 400) Note the swollen columnar cells with many small vacuoles, the brush border was not seriously affected. (bb, brush border; c, columnar cell; g, goblet cell; pm, peritrophic membrane; v, vacuole)

Fig. 83 Transverse section of the midgut epithelium of the fourth instar larva (Parnara guttata) one hour after B. thuringiensis treatment. (P.A.S. stained x 400) Note the mucous layer was not seriously affected (arrowed). (c, columnar cell; g, goblet cell)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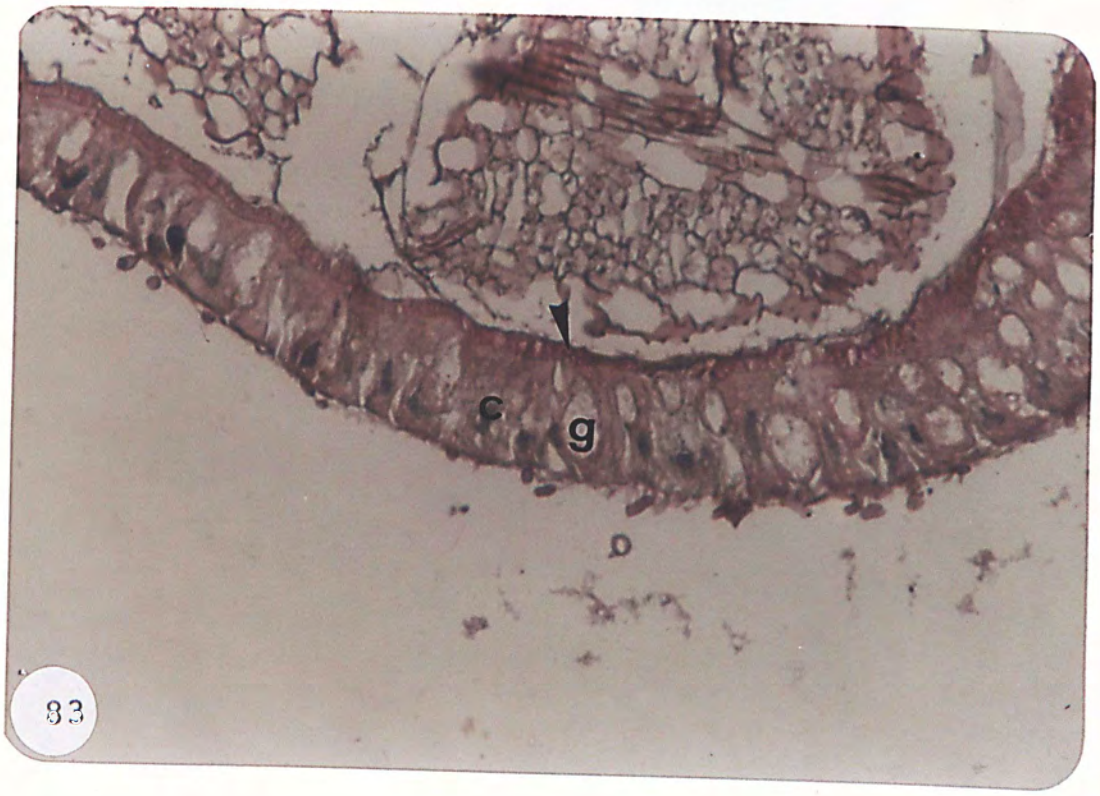
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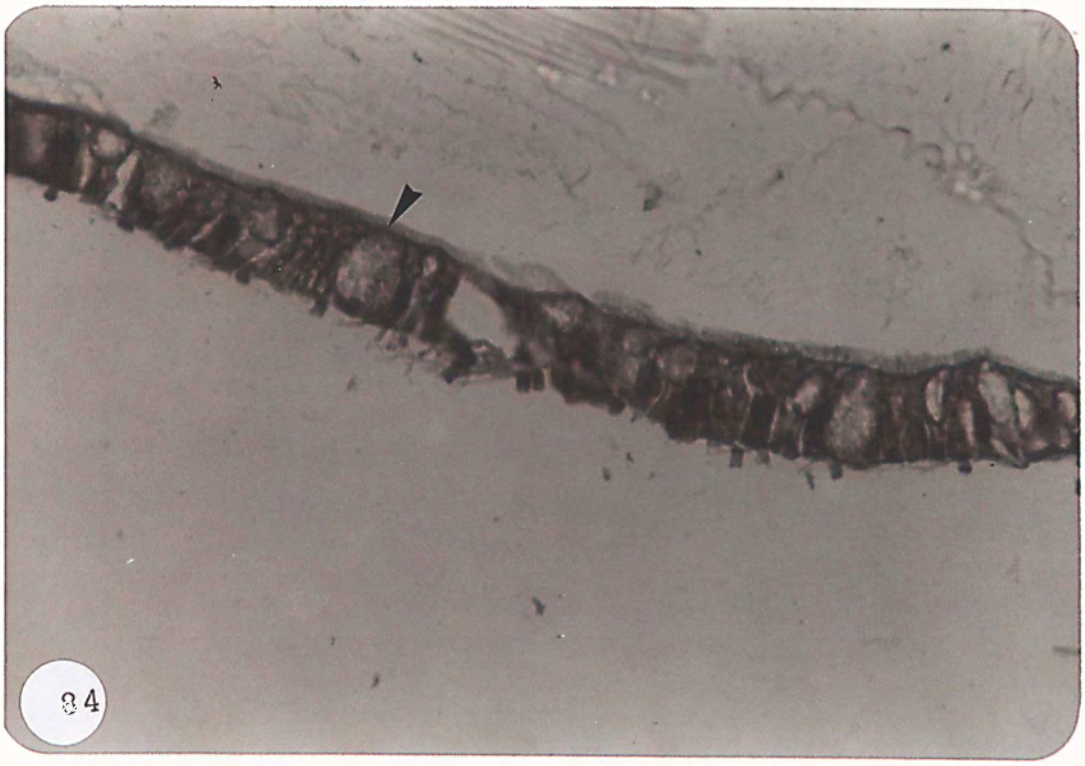
Fig. 84 Transverse section of the midgut epithelium of the fourth instar larva (Parnara guttata) one hour after B. thuringiensis treatment. (Mercury-Bromophenol blue stained x 200) Note the closely packed brush border (arrowed).

Fig. 85 Transverse section of the midgut epithelium of fifth instar larva (Parnara guttata) one hour after B. thuringiensis treatment. (Acid solochrome cyanine stained x 400) Note the high level of basic protein on the epithelial cells (red colour), the nuclei were stained blue (arrowed). (c, columnar cell; d, damaged cell; g, goblet cell)



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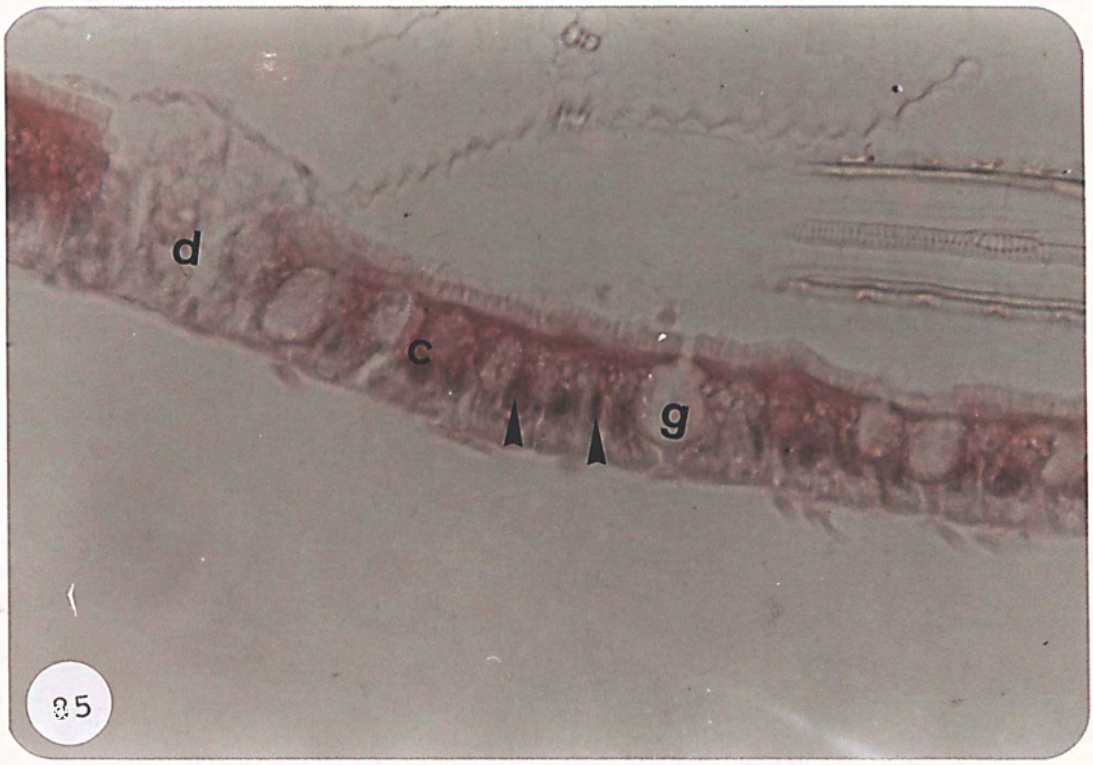

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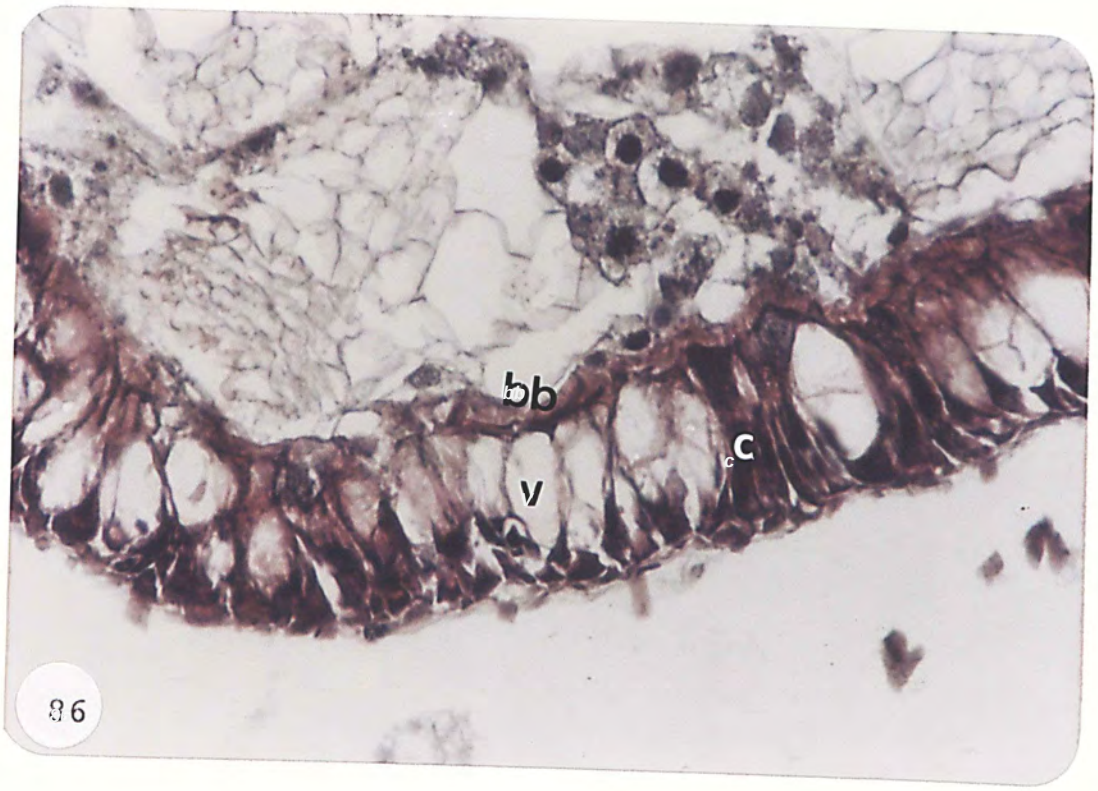
Fig. 86 Transverse section of the midgut epithelium of the fourth instar larva (Parnara guttata) two hours B. thuringiensis treatment. (H & E stained x 400) Note the swollen columnar cells with medium- and large-sized vacuoles in the cytoplasm. (bb, brush border; c, columnar cell; v, vacuole)

Fig. 87 Transverse section of the midgut epithelium of the fourth instar larva (Parnara guttata) two hours after B. thuringiensis treatment. (P.A.S. stained x 400) Note the apices of the columnar cells were extensively stained (arrowed). (v, vacuole)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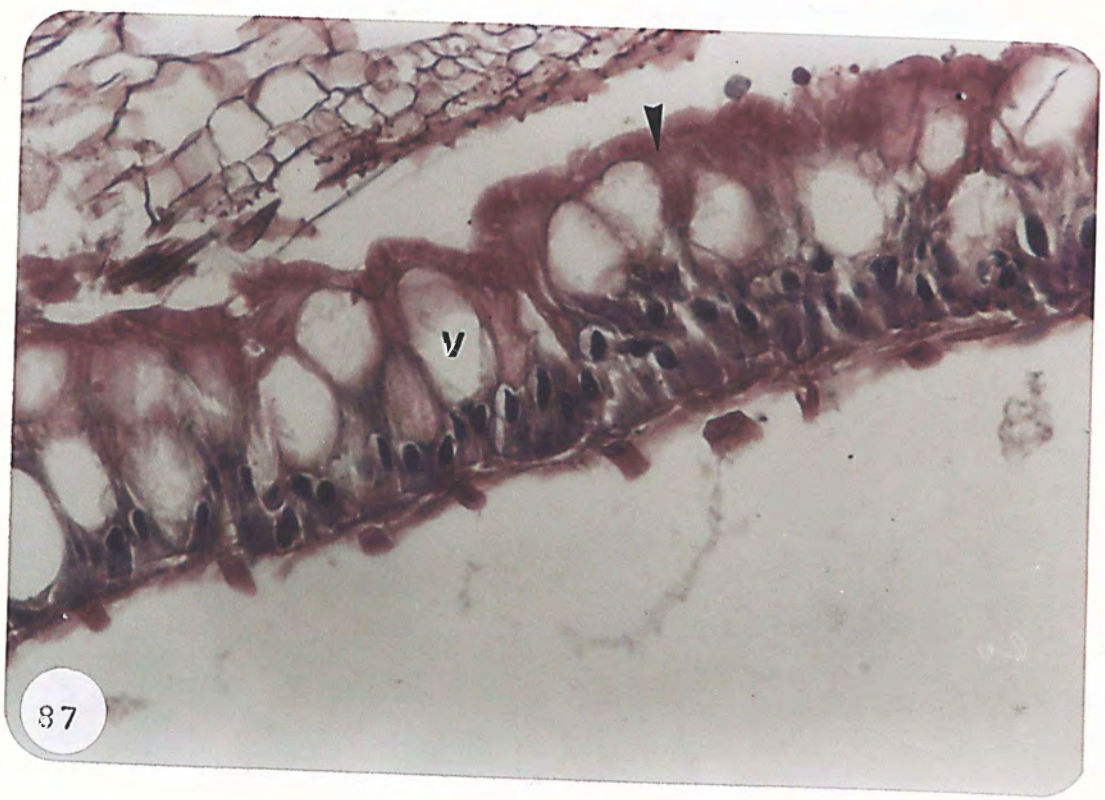
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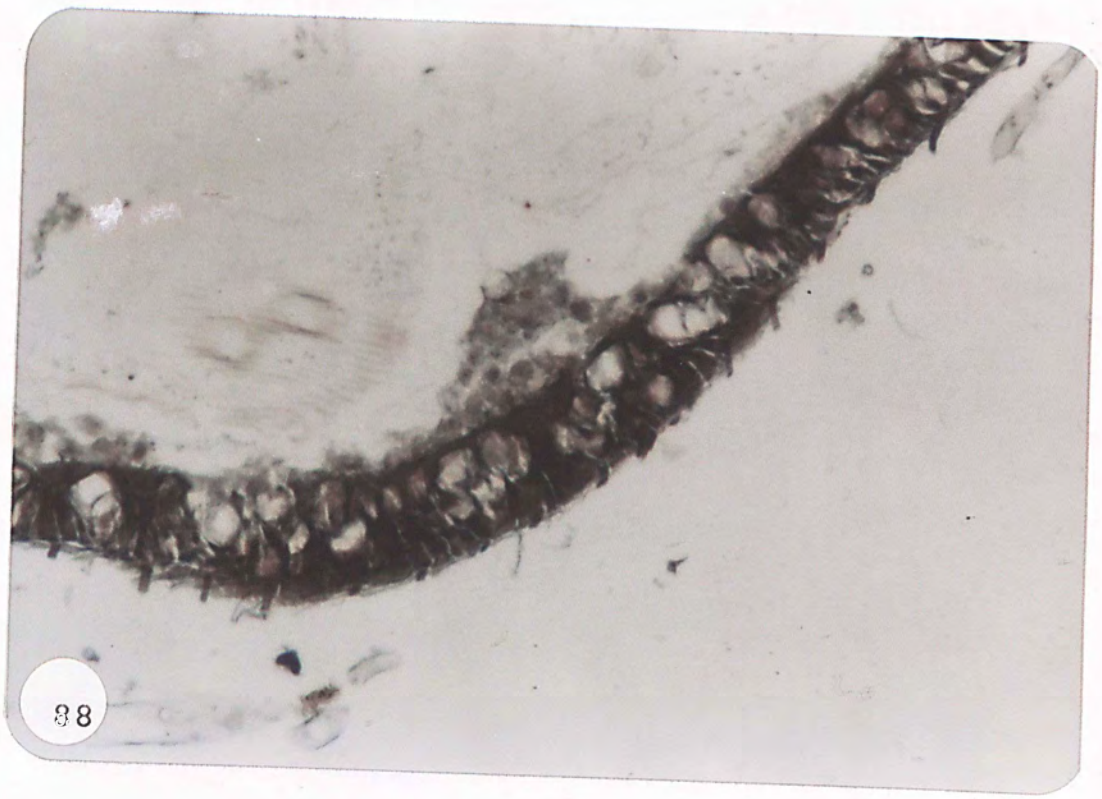
Fig. 88 Transverse section of the midgut epithelium of the fourth instar larva (Parnara guttata) two hours after B. thuringiensis treatment. (Mercury-Bromophenol blue stained x 200) Note the extensive vacuolation.

Fig. 89 Transverse section of the midgut epithelium of fifth instar larva (Parnara guttata) two hours after B. thuringiensis treatment. (Acid solochrome cyanine stained x 400) Note the high level of basic protein on the epithelium (red colour), the nuclei were stained blue. (arrowed). (v, vacuole)



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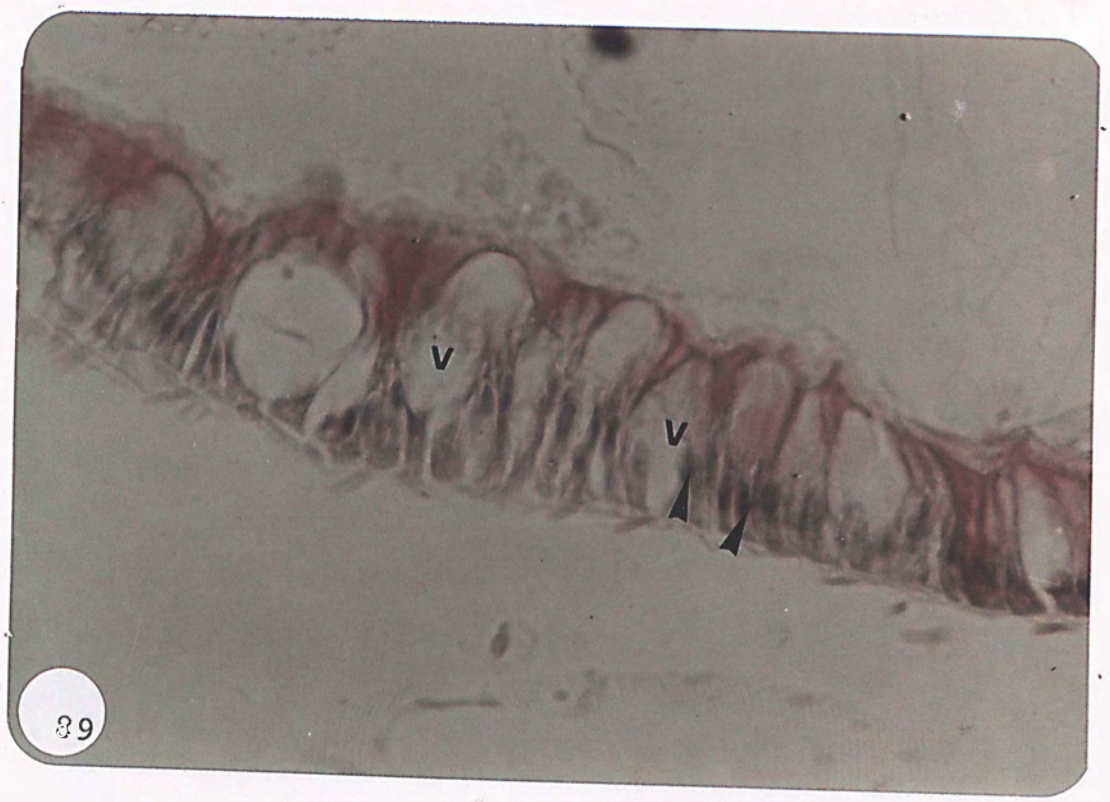

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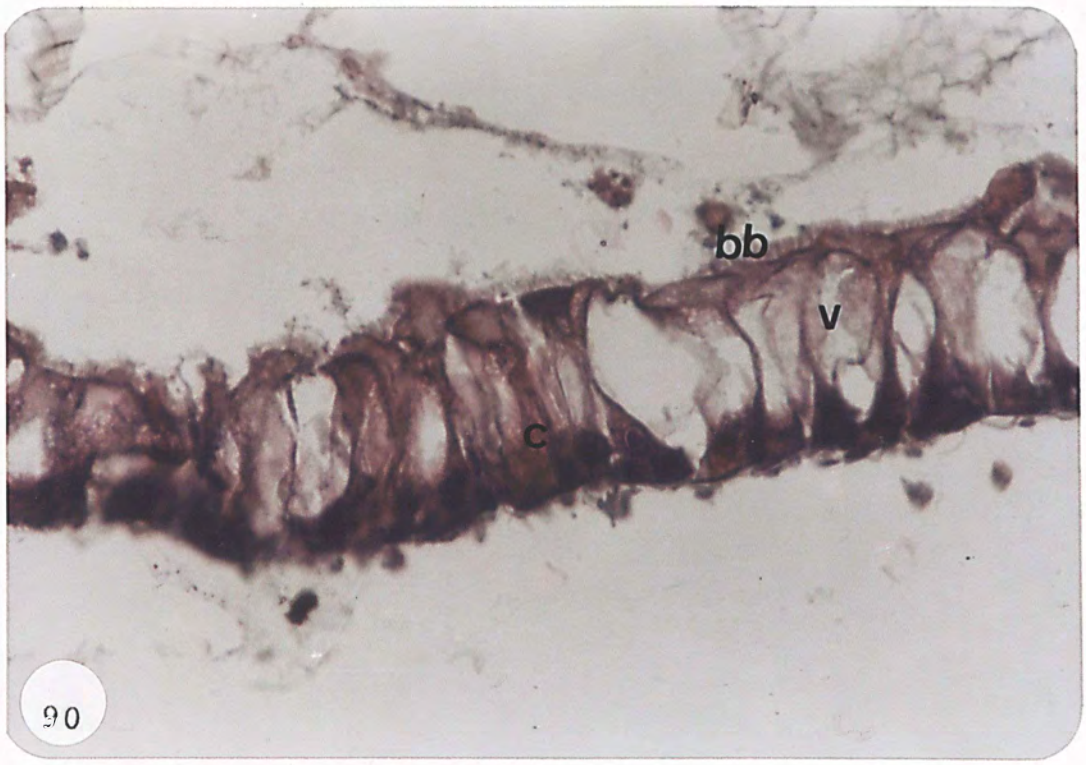
Fig. 90 Transverse section of the midgut epithelium of the fourth instar larva (Parnara guttata) four hours B. thuringiensis treatment. (H & E stained x 400) Note the swollen columnar cells with large-sized vacuoles in the cytoplasm, the brush border was disintegrating. (bb, brush border; c, columnar cell; v, vacuole)

Fig. 91 Transverse section of the midgut epithelium of the fourth instar larva (Parnara guttata) four hours after B. thuringiensis treatment. (P.A.S. stained x 400) Note the apices of the columnar cells were extensively stained (arrowed). (n, nucleus; v, vacuole)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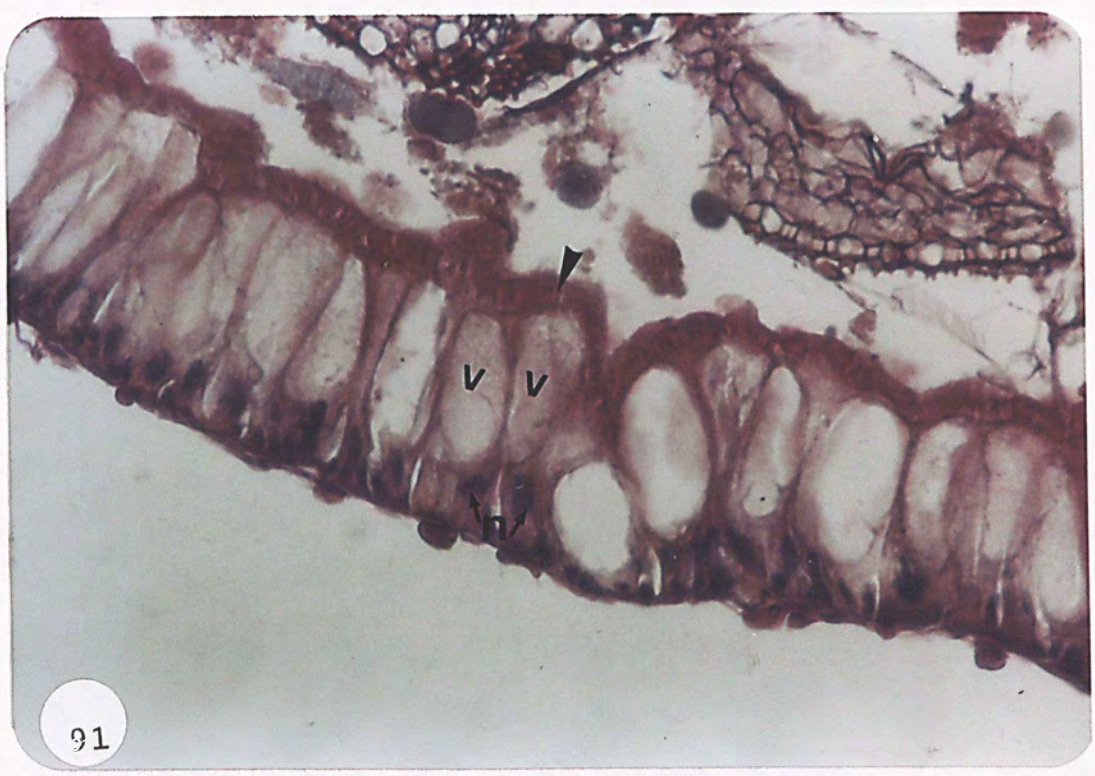
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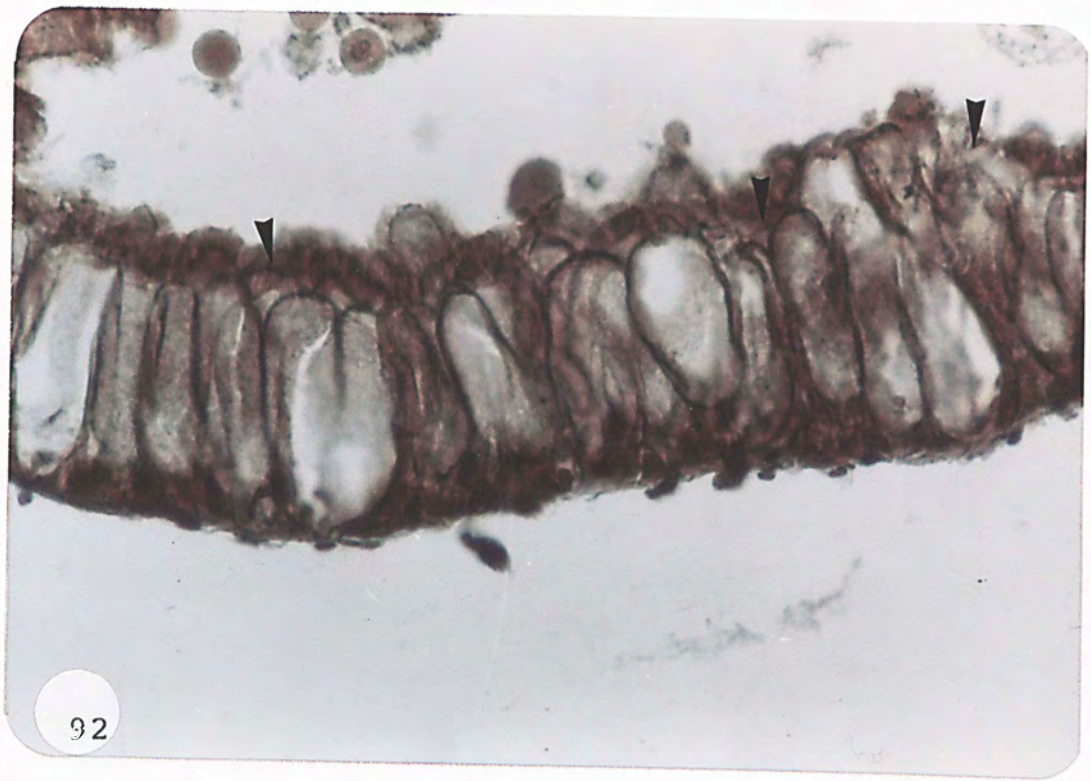
Fig. 92 Transverse section of the midgut epithelium of the fourth instar larva (Parnara guttata) four hours after B. thuringiensis treatment. (Mercury-Bromophenol blue stained x 400) Note the disruption of cell apices (arrowed).

Fig. 93 Transverse section of the midgut epithelium of fifth instar larva (Parnara guttata) four hours after B. thuringiensis treatment. (Acid solochrome cyanine stained x 400) Note the high level of basic protein on the epithelium (red colour), lyses of the cells were observed (arrowed).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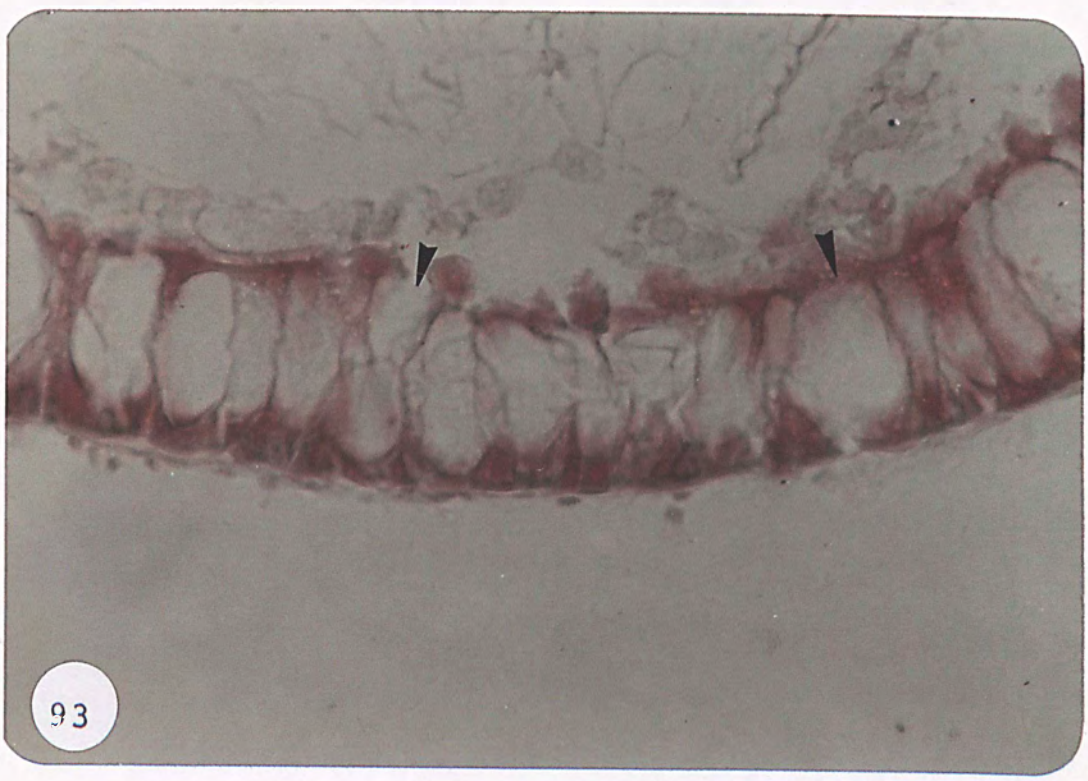
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Fig. 94 Bacteria recovered from the midgut extract of 5-hours-infected larvae (*Pieris canidia*). (Gram stain x 800) Note the rod-shaped, gram-positive bacterial cells arrowed), which were speculated to be *Bacillus thuringiensis*.

Fig. 95 Bacteria recovered from midgut extract of control larvae (*Pieris canidia*) (Gram stain x 800) Note the gram-negative and cocci-shaped bacterial cells.

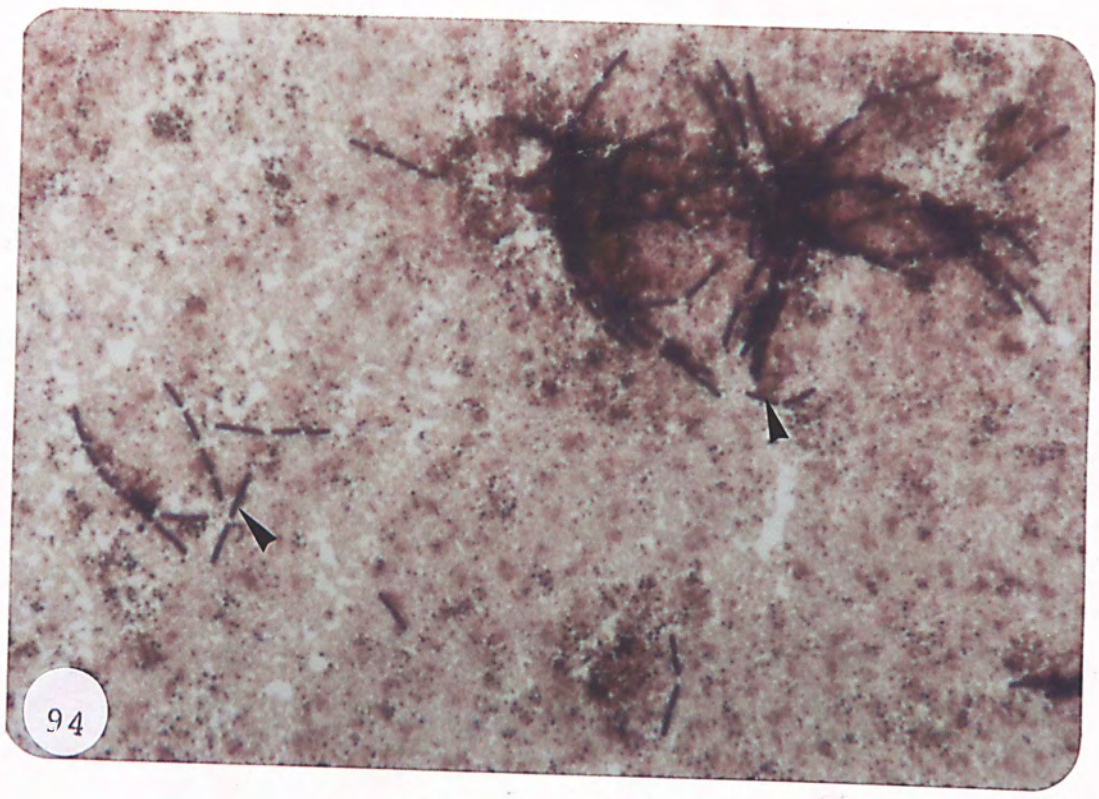
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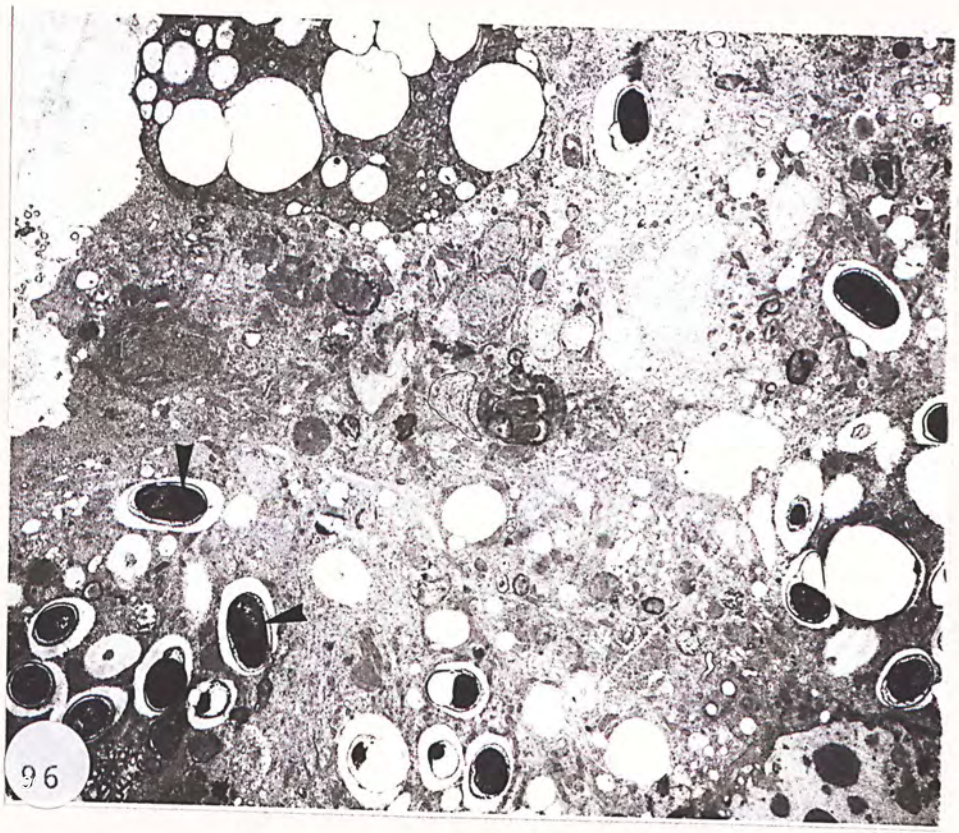
Fig. 96 Epithelial cells of infected larvae (Pieris canidia), being penetrated by bacteria (arrowed), which were speculated to be B. thuringiensis. (TEM x 8280)

Fig. 97 Bacteria found in the gut lumen of infected larvae (Pieris canidia). (TEM x 8280) (ls, longitudinal section of the bacteria; cs, cross section of the bacteria)



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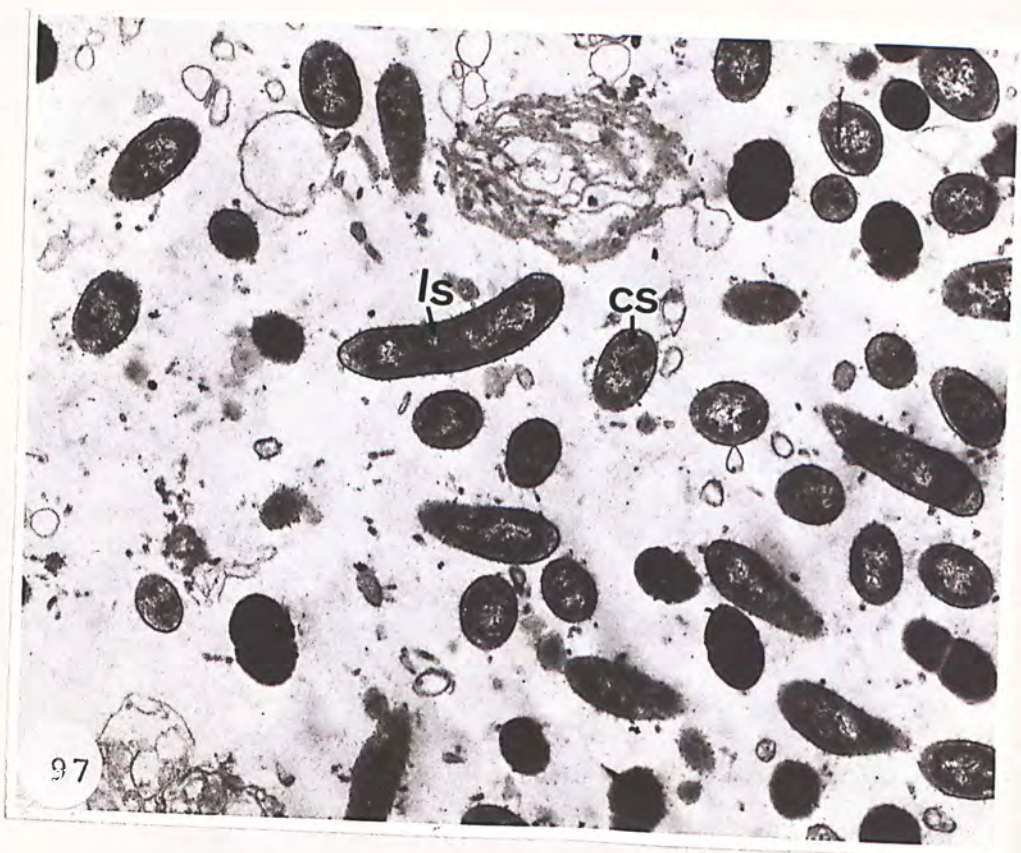

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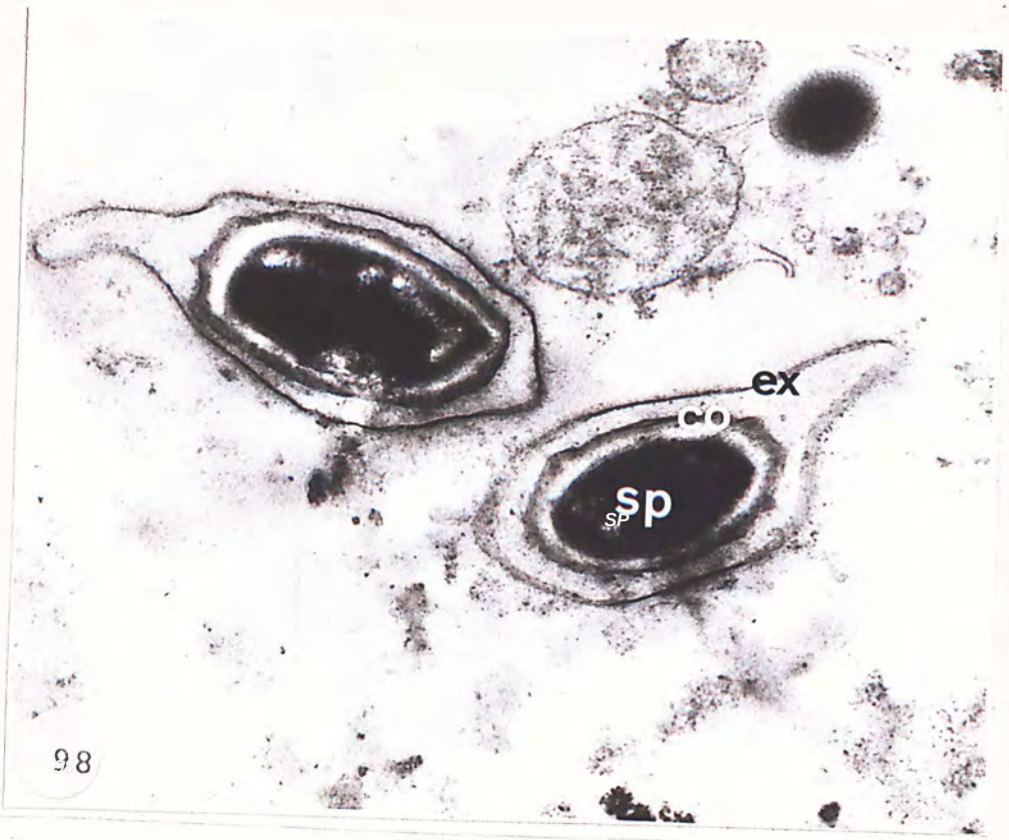
Fig. 98 Bacterial spore found in the gut lumen of infected larvae (Pieris canidia). (TEM x 17280)
(sp, spore; co, cortex; ex, exosporium)

Fig. 99 Bacterial spore (arrowed) found in the gut lumen of infected larvae (Pieris canidia). (SEM x 2400)



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V. DISCUSSION

5.1) Toxic effects in Pieris canidia

a) Effects on the gross structure of columnar cells

The toxic effects on the Pieris canidia midgut wall had been observed 20 minutes, 40 minutes, 1 hour, 2 hours, 3 hours, 4 hours and 5 hours after treatment of B. thuringiensis spore-crystal mixture. Swelling of cells and rapid extrusion of cytoplasm were observed in the columnar cells within 20 minutes. Extrusion of cytoplasm continued in the columnar cells until the columnar cells lysed. Chiang et al (1986) studied the midgut epithelium of the rice moth larva, Corcyra cephalonica, after infection of B. thuringiensis var. kurstaki and observed similar swelling and extrusion of columnar cells within 25 minutes. Endo and Nishiitsutsuji-Uwo (1980) also observed the swelling and extrusion of columnar cells in the silkworm, Bombyx mori, after 30 minutes of B. thuringiensis var. kurstaki and var. aizawai treatment.

b) Effects on subcellular structure of columnar cells

Under the electron microscope, the columnar cells were seriously damaged. The microvilli, which are the first target in contact with B. thuringiensis delta-endotoxin, disrupted in the first hour. Breakdown of membrane permeability might thus be resulted. The endoplasmic

reticulum showed disintegration, small vacuoles were formed simultaneously. The mitochondria became more electron dense. However, the time of occurrence was delayed in Pieris canidia larval midgut. The studies in Bombyx mori indicated that the disruption of endoplasmic reticulum was within 30 minutes (Endo and Nishiitsutsuji-Uwo, 1980), and it took only 5 minutes in Pieris brassica to have deformation of microvilli, swelling of mitochondria and disintegration of endoplasmic reticulum (Ebersold et al, 1977). The discrepencies in efficiency may be the result of different insect species and the bacterial toxins used for the various studies. Ebersold et al (1977) used purified delta-endotoxin, Endo and Nishiitsutsuji-Uwo (1980) used the crystal alone, and in this project the spore-crystal mixture was used. Moreover, the host specificity may proably play an important role in the cellular response to the action of delta-endotoxin.

c) Effects on the goblet cells

The goblet cells were not as seriously affected as the columnar cells. In Bombyx mori, the goblet cells responded slightly under the infection of B. thuringiensis delta-endotoxin (Endo and Nishiitsutsuji-Uwo, 1980). Under electron microscopic studies, the goblet cells were indeed slightly affected. The appearance of granular and electron dense materials in the cytoplasm of goblet cells indicated that the active transport was probably impaired such that

ions accumulated in the cytoplasm (Endo and Nishiitsutsuji-Uwo, 1980). The goblet cavities were also found to be enlarged in silkworm epithelium after intoxication of B. thuringiensis. The goblet cavity swelled in the larval midgut epithelium in Pieris canidia three hours after intoxication, however the response in goblet cells was far less serious than that of the columnar cells. The swelling of goblet cells could be explained by the breakdown of active transport, which led to the accumulation of K+ ions in the goblet cells. An increase of ion concentration resulted in the decrease of water potential within the cell. Water was thus drawn into the goblet cells through the water potential gradient, consequently, the goblet cells were swollen up.

d) Defense reaction

In the present study, secretory products were found at the apices of the columnar cells and in the goblet cavity in Pieris larvae. They could be considered as a kind of defensive mechanism as proposed by other workers (Chiang et al, 1986) to protect the insect midgut from bacterial infection (Fast and Angus, 1965). The appearance of defensive secretion prolonged the survival time of the larvae (Chiang et al, 1986). At the third hour after intoxication, the defensive secretion stopped and the mucous

layer became thinner and even completely lost eventually. This might be probably due to the lysis of the columnar cells in the midgut epithelium. The responses were quite simliar to the mucous layer studied by Chiang et al (1986) in infected Corcyra larval midgut. They observed the presence of chalice-shaped vesicles which were believed to be defence secretions. The second type of defense mechanism in the larval midgut is the replacement of morbid cells by the regenerative cells. In Pieris canidia larvae, the regeneration took place within the first hour. The replacement of the damaged columnar cells prolonged the survival of the midgut epithelial cells after the action of B. thuringensis delta-endotoxin. However, those newly formed columnar cells suffered from the actions of the toxin as well. As a result, swelling and lysis of the newly formed cells were observed in subsequent hours. Breakdown of midgut epithelium was thus resulted.

e) Histochemical changes

Three major cellular components were found to have changed dramatically in the epithelium. They were the basic protein level, the lipid content, and the activity of alkaline phosphatase.

i) Basic protein

Immediately after intoxication of B. thuringiensis spore-crystal mixture, the basic protein level increased and maintained at a high level until the gut epithelium disrupted. The high level of basic protein indicated that the pH in the gut wall was high after intoxication. This high pH enabled solubilization of protoxin crystal in the insect gut (Bulla et al, 1977), therefore sustaining the activation of delta-endotoxin.

ii) Lipid content

The lipid, mainly the phospholipid level in larval midgut epithelium of Pieris canidia was seriously affected by B. thuringiensis delta-endotoxin. Phospholipids are one of the major compound lipids present in the epithelial cells of Pieris larvae (Turnnen, 1973). After intoxication, the lipid content was completely lost in the midgut epithelium. This may imply the impairment of lipid uptake and/or the exhaustion of lipid storage in the midgut tissue.

iii) Alkaline phosphatase avtivity

The alkaline phosphatase tested in this project is the non-specific alkaline phosphatase, which hydrolyses phosphate esters and ensures phosphate transfer (Pearse, 1968). In Pieris larvae, the activity of alkaline phosphatase declined after intoxication and increased in the first hour when regeneration of columnar cells took place. The activities of alkaline phosphatase were completely lost after cell lysis. As the activity of alkaline phosphatase is mainly located at the apical side of the columnar cells in association with the brush broder, the delta-endotoxin induced extrusion of cytoplasm resulted in the decline of the alkaline phosphatase activity. The enzymes were probably resynthesised as regeneration of columnar cells took place, and they were transported to the apical region of the newly formed columnar cells. Lysis of the cells in the fourth hours resulted in the complete loss of alkaline phosphatase activity.

5.2) Toxic effects in Parnara guttata

a) Histopathology

In Parnara guttata larvae, the responses of epithelial cells after infection of B. thuringiensis delta-endotoxin differ from the Pieris larvae greatly. No extrusion of cytoplasm was observed in the columnar cells, but swelling of the columnar cells and increase in vacuolation were observed in the first hour. No regeneration of the columnar cells was demonstrated in Parnara larval midgut. The size of columnar cells increased as time went by. The small vacuoles associated to form a medium-sized vacuole located around the centre of a morbid cell. The nucleus was thus pushed to the basal region of the columnar cell. The vacuoles expanded in the fourth hour and the increase of osmotic pressure broke the cell membranes of the infected cells which resulted in cell lysis.

b) Vacuolation

The formation of vacuoles is probably due to the breakdown of active transport of ions in the midgut tissue. Under normal conditions, ions and nutrients from the gut lumen enter the midgut epithelial cells by diffusion at the microvilli of the apical side. These ions and nutrients

accumulated in the extracelluar sinuses at the basal side of the columnar cells by active transport (Treherne, 1967). As a result, a low water potential is generated in the extracellular sinuses. Thus, water from both the luminal and the haemocoel side (Treherne, 1967) enters the extracelluar space by rapid osmosis. A water current is formed to carry the ions and nutrients from the extracellular space to the haemocoel (Berridge, 1970). After intoxicated by delta-endotoxin , the active transport function of the epithelium was lost. Thus the osmotically active molecules can only enter the columnar cells by diffusion. The cytoplasm of the cells would then be hypertonic to the extracellular space. As a result, water was drawn into the epithelial cells to form vacuoles which would eventually lead to osmotic damage of cell.

5.3) Difference in responses between *Pieris* and *Parnara* larvae

Comparing the effects of *B. thuringiensis* spore-crystal mixture on the two insect larvae tested, some differences in the cellular response were noted.

a) Extrusion of cytoplasm

The extrusion of cytoplasm is a common feature after the B. thuringiensis delta-endotoxin treatment in various insects, such as Bombyx mori (Endo and Nishiitsutsuji-Uwo, 1980) and Corcyra cephalonies (Chiang et al, 1986). In Pieris canidia, extrusion of cytoplasm happened 20 minutes after B. thuringiensis treatment, however, no rapid extrusion of cytoplasm was observed in Parnara guttata midgut epithelial cells until lysis of the cells happened. This is probably due to the different responses of the two insects to the intoxication of B. thuringiensis delta-endotoxin. It is postulated that the osmotic balance in the epithelial cells of Parnara larvae were altered, which resulted in a rapid uptake of water into the cells.

b) Regeneration of the epithelial cells

The morbid cells caused by the intoxication of B thuringiensis delta-endotoxin might lose their normal functions, and the regenerative cells develop to replace them (Chiang et al, 1986). This replacement of damaged cells were observed in Pieris larvae, but not in Parnara larvae. It may be due to the fact that the surface inhibition limited the growth of the un-differentiated cells. As the columnar cells were damaged, they extruded

their cytoplasm continuousely as shown by exocytosis or merocrine secretion. As a result, the damaged columnar cells released the surface inhibition of the near-by undifferentiated cells and promoted their proliferation. In Parnara, the cases were different, the morbid cells neither extruded their cytoplasm nor lysed at the early stage, surface inhibition on the regenerative cells was relatively high. Therefore, no regeneration was observed in Parnara larval midgut after infection of B. thuringiensis.

c) Vacuolation in cytoplasm

Vacuolation is a general pathological observation on the epithelial cells after the infection of B. thuringiensis. In Pieris larvae, numerous small vacuoles were formed in the cytoplasm and the vacuoles remained in similar forms until the cell lysed. However, in Parnara larvae, the small vacuoles grew up as time went by, until a centrally located large vacuole was observed in the fourth hour after B. thuringiensis treatment. This difference might be a result of dissimilar response in these two insect species. The Pieris larvae extruded its cytoplasm as a rapid defense response. In Parnara epithelial cells, damages of cells arose slowly as a result of formation of large vacuoles. Eventually, the osmotic damages lead to cell lysis.

d) Brush border

The brush border was speculated to be the first target of delta-endotoxin, and was found to be disrupted in various insects early after infection of B. thuringiensis (Oron et al, 1985). In Pieris canidia larvae, the brush border of the columnar cells disrupted within the first hour after B. thuringiensis treatment. In Parnara, the brush border was apparently less affected by the B. thuringiensis delta-endotoxin even at the fourth hour after B. thuringiensis treatment. Since electron microscopic study had not been carried out in Parnara owing to the shortage of material supply, it is difficult to conclude, at this stage, that whether there was also any damage of Parnara midgut microvilli by B. thuringiensis delta-endotoxin.

Generally speaking, the difference in cellular responses was due to host specificity of the bacterial toxin (Lecadet and Martouret, 1987). Studies of the toxicity of different B. thuringiensis strains on Pieris brassicae and Spodoptera littoralis showed that the LD . varied greatly in the same insect after exposure to different strains of B. thuringiensis. They proposed that the endotoxins were host specific. Indeed, there are variations in the amino acid sequence and different sizes of the active units in endotoxins produced by various strains of bacteria.

VI. CONCLUSION

The effects of B. thuringiensis spore-crystal were on the larval midgut epithelium of Pieris canidia and Parnara guttata. The infection caused damages in the epithelial cells. It was found that the columnar cells were the most susceptible cell type to B. thuringiensis delta-endotoxin. Swelling, vacuolation and extrusion of cytoplasm were the general effects caused by the delta-endotoxin. The damaged cells then lysed and the gut epithelium disrupted. Subsequently, there was mixing-up of the gut fluid and the haemolymph, resulting in increase of the pH of the latter. The insect would later die of alkalosis.

However, the cellular responses in the two tested insect larvae were different. In Pieris canidia, the columnar cells of the infected midgut epithelium were damaged in the first 20 minutes. Rapid extrusion of cytoplasm and vacuolation were the first predominant responses noted, with regeneration of epiithelial cells noted within one hour. Secretions were observed in the epithelial cells which finally lysed four hours after treatment. Only a sparse layer of cuboidal cells was found lining the midgut epithelium then. The function of these cells was not known. Eventually, the larvae died.

The responses in Parnara larvae were different from that of Pieris larvae. Vacuolation was the prominant response observed in the epithelium, neither extrusion of cytoplasm nor regeneration of morbid cells were observed. Cells lysed due to osmotic damages.

The cellular responses in Pieris canidia observed were highly compatible to the reports by Endo and Nishiitsutsuji-Uwo (1980) on the silkworm, Oron et al. (1985) on the cotton leaf worm, and Chiang et al. (1986) on the moth borer. However, the time of appearance of the toxic responses in Pieris canidia and the different responses in Parnara guttata were probably due to the specificity in B. thuringiensis var. kurstaki (Lecadet and Martouret, 1987).

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VIII. APPENDIX 1

1) Histopathological studies:

1.1) B.t. preparation:

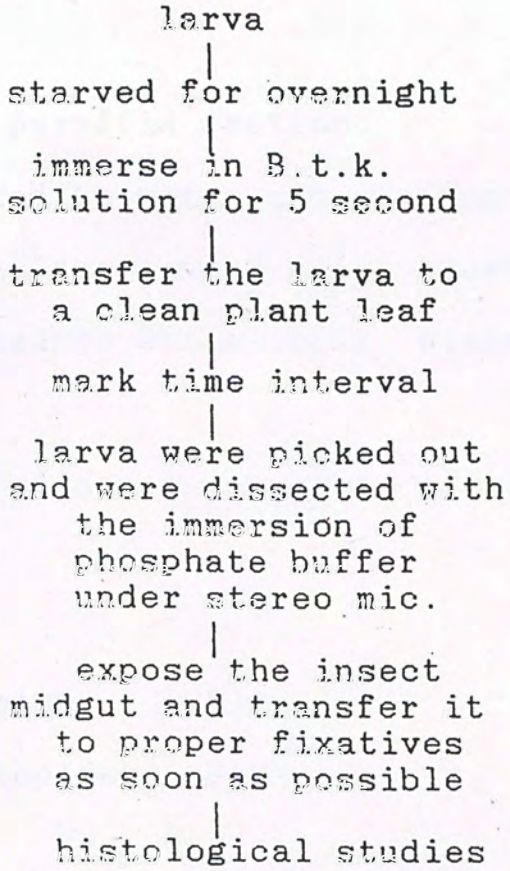
- B.t. : Bacillus thuringiensis var. kurstaki (B.t.k.) (Thuricide, Sandoz), mixture of spores and crystals 16000 IU per gram, commercial formulation: 25g B.t. in one 1 L water

- concentration used, 2.5 %, w/v.

- Route of administration:
  Immerse insect larva in 50 ml B.t. solution (2.5%, w/v)
  The B.t. will get into the larva orally

- larvae: Pieris canidia, Parnara guttata

- Procedure:



Histological studies:

a) Hematoxylin and Eosin Method

Fixation : Bouin's solution

Phosphate buffered formalin

Embedding : Paraffin wax

Section : 5 µm sections by microtome

Procedure:

1) Dewaxed paraffin section
2) Rehydrated to water and stained in Hematoxylin (Mayer's solution) for 1 to 2 min., blueing in running water.
3) Dehydrated to 95% alcohol, stained with eosin (1%) for 20-30 second.
4) Put the slides in absolute alcohol, clear in xylene, mount in balsam.

Result: Nuclear - Blue

Cytoplasm - Pink

b) Toluidine Blue Method:

Fixation: Glutaraldehyde-Osmium tetroxide (thick section)

Embedding: Spurr´s medium

Section: 1 µm thick section

Procedure:

1) Section on slide flood with 1% toluidine blue (in 1% borax buffer) for 1.5 to 2 min.
2) Washed in running water
3) Examine under microscope

c) Mercury-Bromophenol Blue Method

Fixation: Bouin's fixatives

Buffered Formalin

Embedding: Paraffin wax

Section: 5 µm Paraffin section

Mercury-Bromophenol Blue soluton:

Dissolved 0.4 gm $HgCl_2$ in 40 ml of 2 % acetic acid, and added 20 mg bromophenol blue.

Procedure:

1) Dewaxed section to water
2) Stained in Mercury-Bromophenol Blue for 30 min
3) Rinse sections in 0.5 % acetic acid, 3-5 min
4) Dehydrated in tertiary butyl alcohol
5) Clear in xylene amd mount in balsam

Result: Protein - Deep blue

d) Acid Solochrome Cyanine Method:

Fixation: Bouin's fixatives

Embedding: paraffin wax

Section: 5 µm paraffin section

Procedure:

1) Dewaxed tissue rehydrated to water
2) Stained in Acid Solochrome Cyanine (1% in 1% H_3PO_4)
   for 20 min.
3) Washed in water
4) Dehydrated in alcohol, clear in xylene, and mount in balsam.

Result: Nuclear materials - Blue
Basic protein - Red

e) Periodic Acid Schiff Method (PAS method)

Fixation: Bouin´s fixatives

Embedding: paraffin wax

Section: 5 µm paraffin section

Solutions: 1) Schiff´s reagent (de Thomasi)

Basic fuchsin	1	g
Hydrochloric acid (1N)	20	ml
Sodium metabisulphite	1	g
Distilled water	200	ml

2) Sulphite rinses

Potassium metabisulphite (10%)	7.5 ml
Hydrochloric acid (1N)	7.5 ml
Distilled water	135 ml

Procedure:

1) Dewaxed tissue rehydrated to water,
2) Oxidize for 10 minutes in 1% aqueous periodic acid,
3) Wash in running water for 5 minutes, and rinse in distilled water,
4) Imersed in Schiff reagent for 60 minutes,
5) Transfer directly to first sulphite rinse for 1 minute,
6) Transfer directly to second sulphite rinse for 2 minutes,
7) Transfer directly to third sulphite rinse for 2 minutes,
8) Wash for ten minutes in running water,
9) Counterstain with hematoxylin,
10) Dehydrate, clearin xylene, and mount in balsam.

Results: P.A.S. positive materials - bright red
nuclei - blue

f) Alcian Blue Method

Fixation: Bouin's fixatives

Embedding: paraffin wax

Section: 5 μm paraffin section

Procedure:

1) Dewaxed tissue rehydrated to water,
2) Stained in Alcian Blue for 20 minutes,
3) Rinse in distilled water,
4) Counterstain with 0.1% safranin for 30 seconds,
5) Rinse in water and dehydrate rapidly in 95% and absolute alcohol,
6) Clear in xylene, and mount in balsam.

Result: Acid mucin, and most
Sulphated mucopolysaccharide - blue
Other tissue constituents - red

g) Sudan Black Method

Fixation: Fresh frozen tissue

Formol- Calcium in 4°C overnight

Section: cryostat section (10 µm)

Solution: Sudan Black solution

Sudan Black B	7 gm
70 % alcohol	500 ml

Add the dye to the alcohol and warm to 56°C, stand for 1 hour, store in a tightly stoppered bottle when cool. Filter before use.

Procedure

1) Section to 70 % alcohol
2) Stain in Sudan Black for 10 min
3) Rinse in 70 % alcohol
4) Wash in water
5) Mount in glycerine gelatine

Result: Lipids - black

Other tissue - greyish